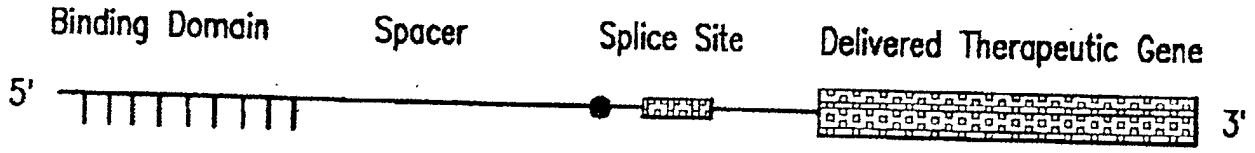

Binding Domain
Spacer
Splice Site
Delivered Therapeutic Gene
5'
3'

FIG.1A

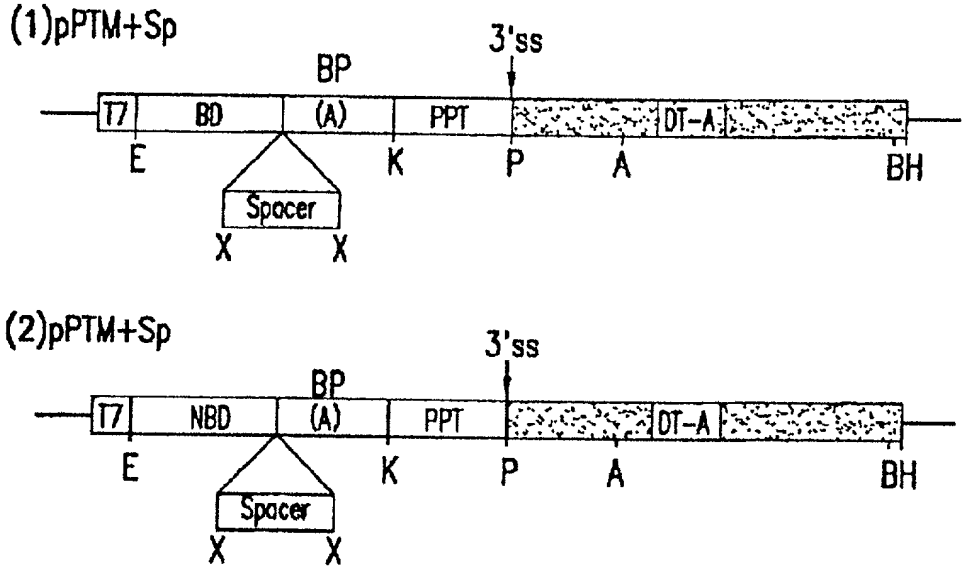

(1)pPTM+Sp
3'ss
BP
T7
BD
(A)
PPT
DT-A
E
K
P
A
BH
Spacer
X
X
(2)pPTM+Sp
3'ss
BP
T7
NBD
(A)
PPT
DT-A
E
K
P
A
BH
Spacer
X
X

FIG.1B

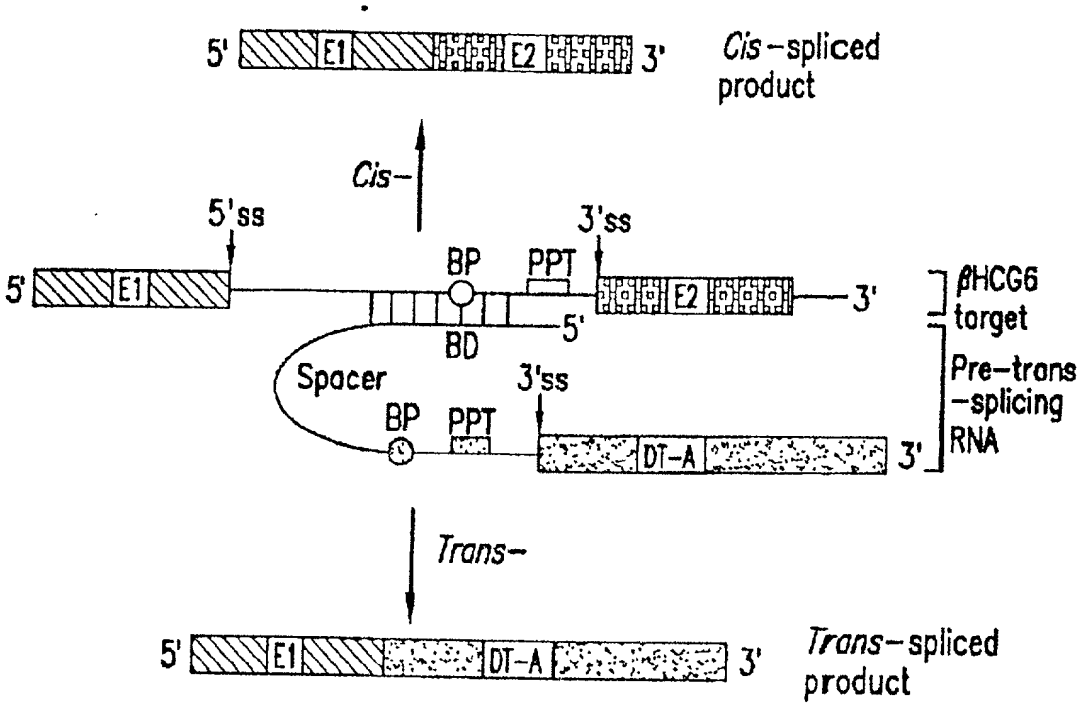

5'
E1
E2
3'
Cis-spliced product
Cis-
5'ss
3'ss
BP
PPT
5'
E1
E2
3'
βHCG6 target
BD
Spacer
3'ss
BP
PPT
DT-A
3'
Pre-trans-splicing RNA
Trans-
5'
E1
DT-A
3'
Trans-spliced product

FIG.1C

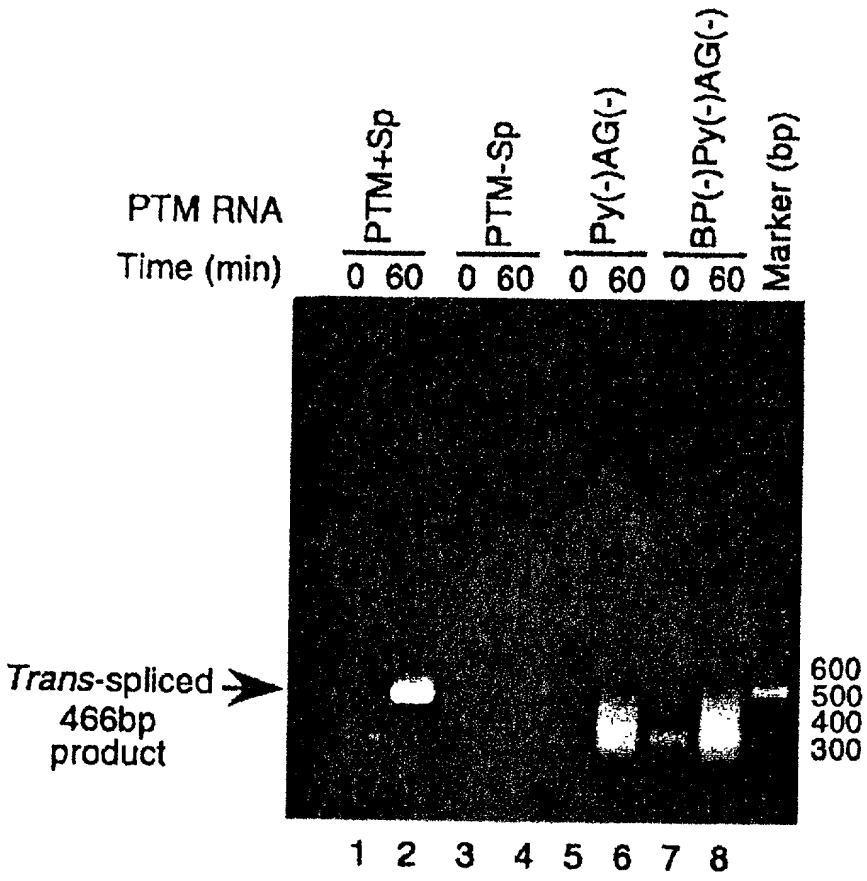

PTM RNA
Time (min)
PTM+Sp
PTM-Sp
Py(-)AG(-)
BP(-)Py(-)AG(-)
Marker (bp)
0 60 0 60 0 60 0 60
Trans-spliced
466bp
product
600
500
400
300
1 2 3 4 5 6 7 8

FIG.2A

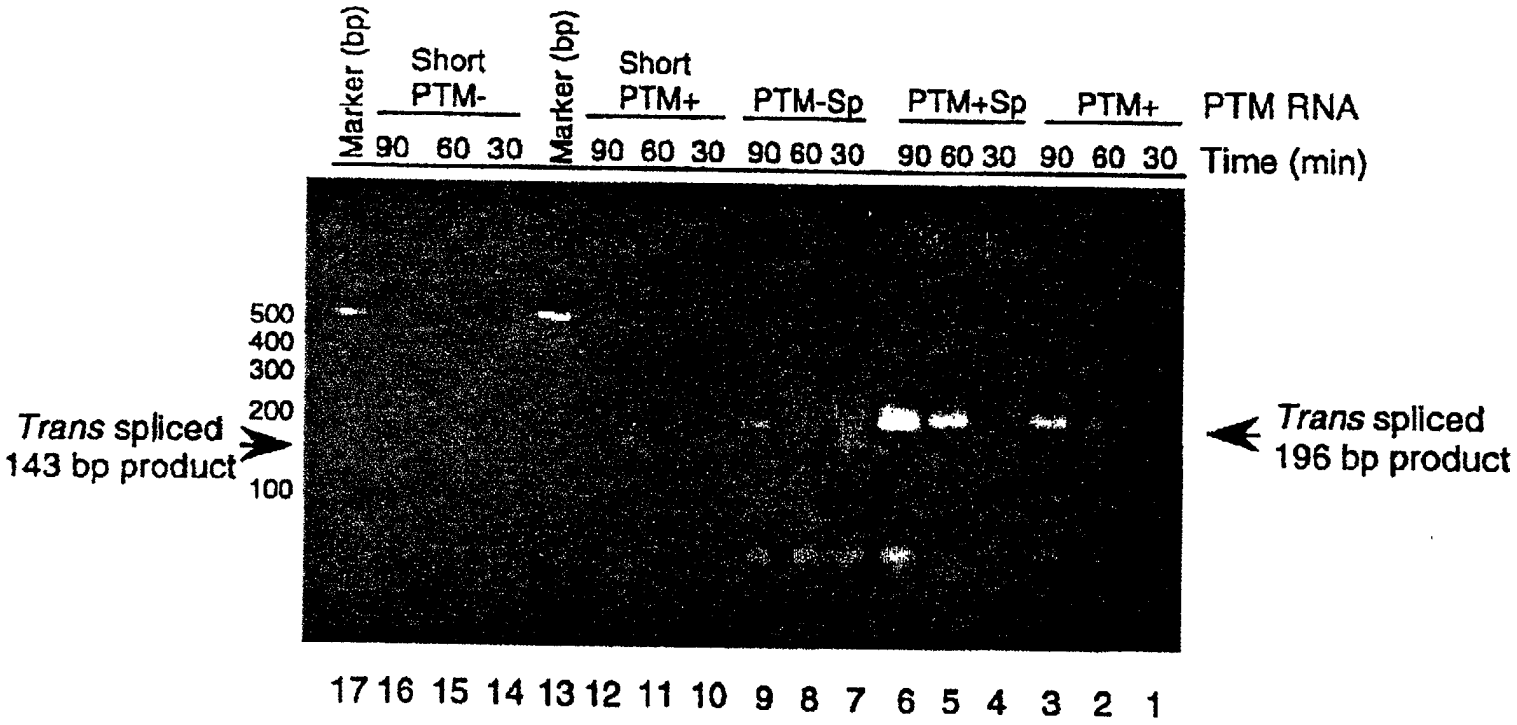

Marker (bp)
Short PTM-
Marker (bp)
Short PTM+
PTM-Sp
PTM+Sp
PTM+
PTM RNA
90 60 30 90 60 30 90 60 30 90 60 30 90 60 30 90 60 30
Time (min)
500
400
300
200
100
Trans spliced 143 bp product
Trans spliced 196 bp product
17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

FIG.2B

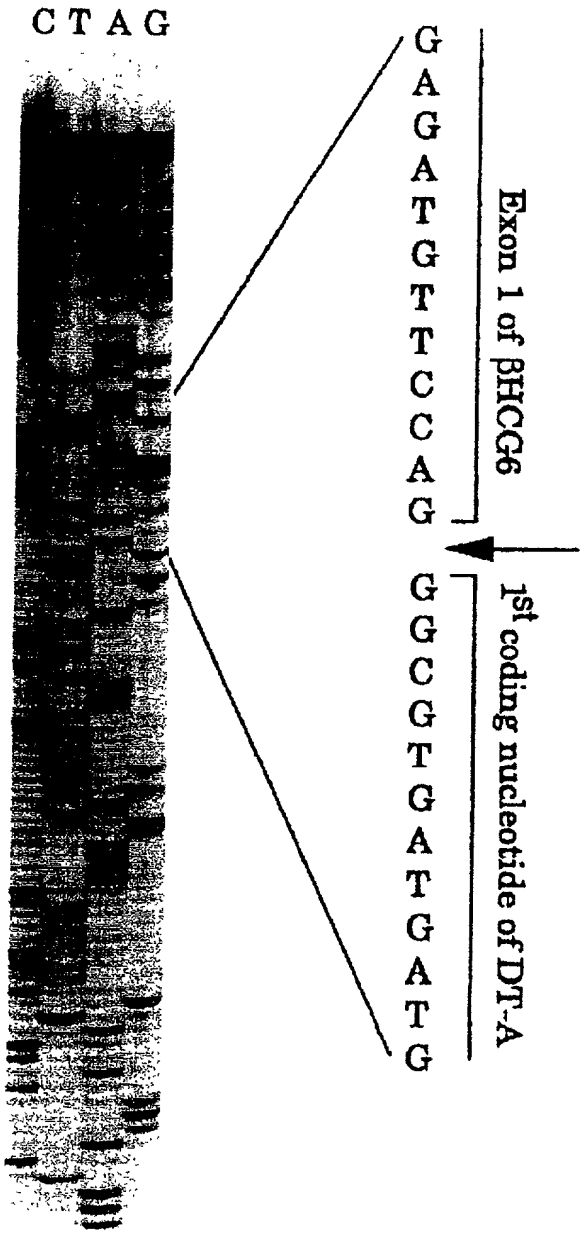
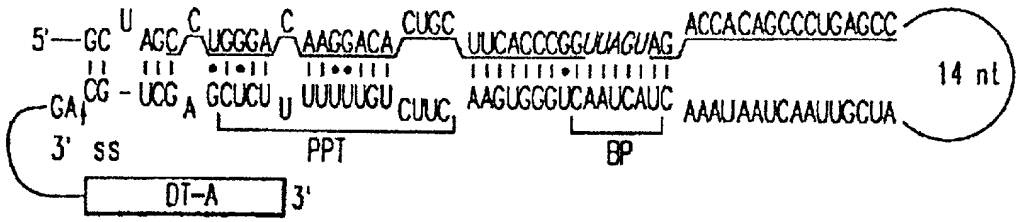
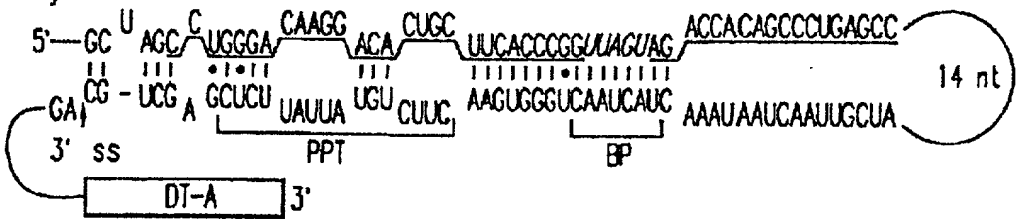

C T A G
G
A
G
A
T
G
T
T
C
C
A
G
Exon 1 of βHCG6
1st coding nucleotide of DT-A
G
G
C
G
T
G
A
T
G
A
T
G

FIG.3

1.PTM+SF:



2.PTM+SF-Py1:



3.PTM+SF-Py2:

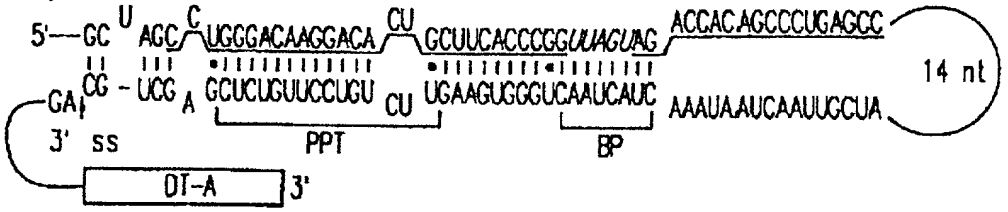


FIG.4A

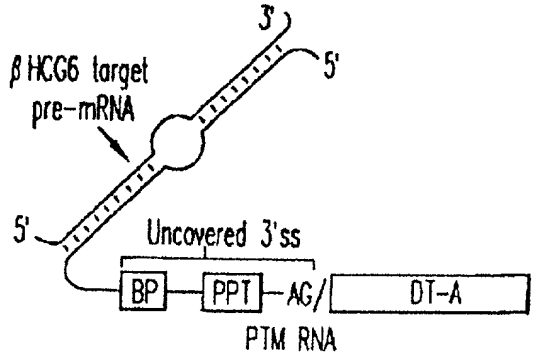


FIG.4B

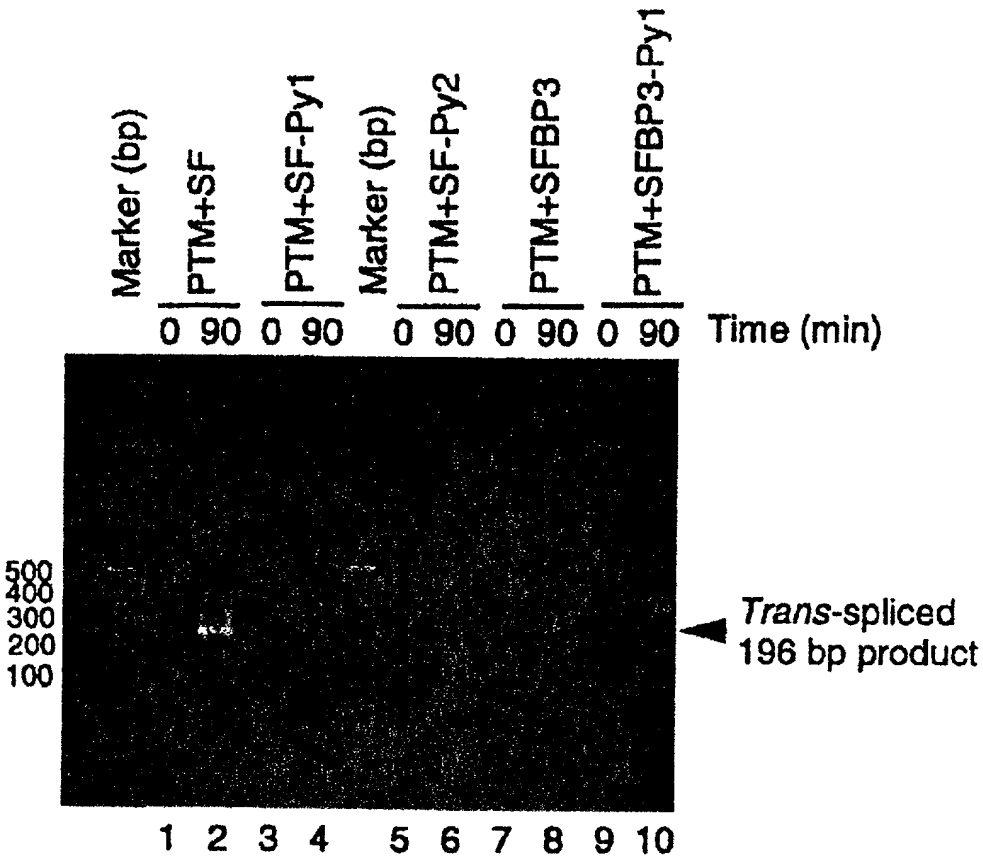

Marker (bp)
PTM+SF
PTM+SF-Py1
Marker (bp)
PTM+SF-Py2
PTM+SFBP3
PTM+SFBP3-Py1
0 90 0 90 0 90 0 90 0 90
Time (min)
500
400
300
200
100
Trans-spliced
196 bp product
1 2 3 4 5 6 7 8 9 10

FIG.4C

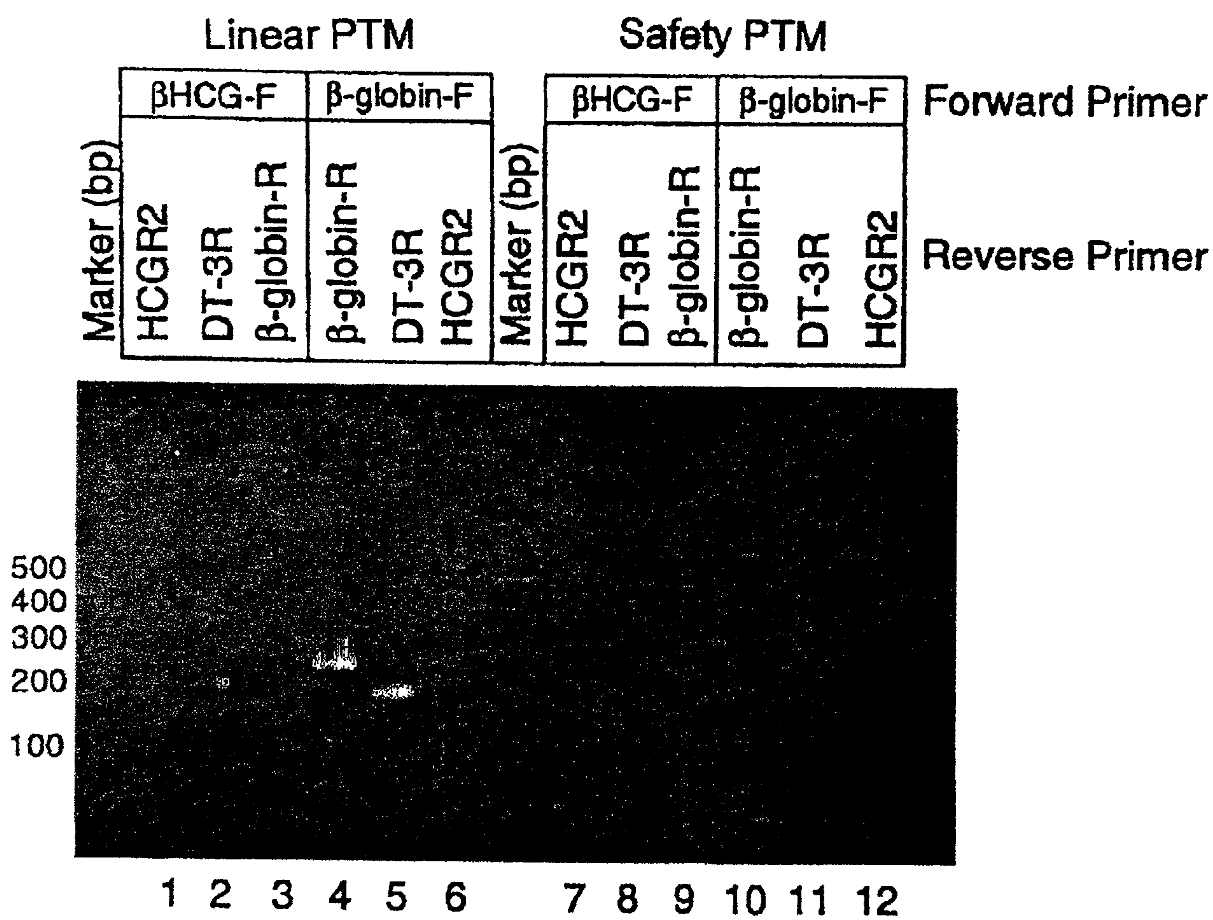

Linear PTM
Safety PTM
βHCG-F
β-globin-F
βHCG-F
β-globin-F
Forward Primer
Marker (bp)
HCGR2
DT-3R
β-globin-R
β-globin-R
DT-3R
HCGR2
Marker (bp)
HCGR2
DT-3R
β-globin-R
β-globin-R
DT-3R
HCGR2
Reverse Primer
500
400
300
200
100
1 2 3 4 5 6 7 8 9 10 11 12

FIG.5

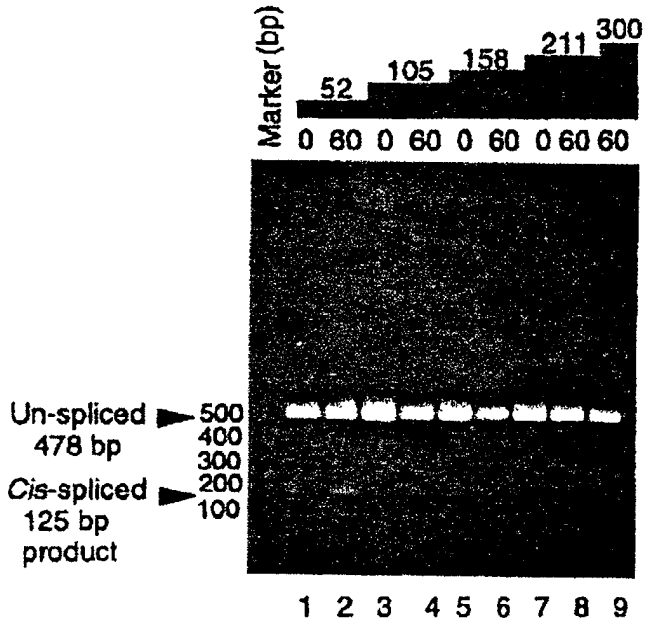

Marker (bp)
52
105
158
211
300
0 60 0 60 0 60 0 60 60
Un-spliced
478 bp
Cis-spliced
125 bp
product
500
400
300
200
100
1 2 3 4 5 6 7 8 9

FIG.6A

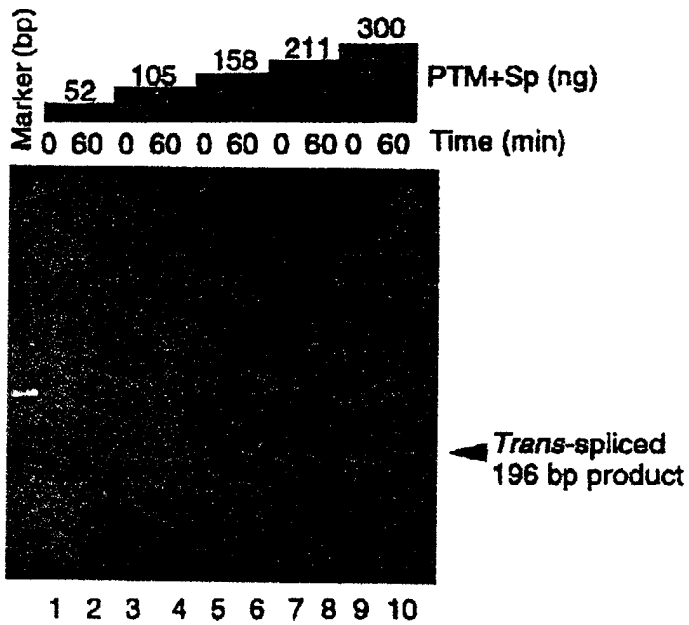

Marker (bp)
52
105
158
211
300
PTM+Sp (ng)
0 60 0 60 0 60 0 60 0 60
Time (min)
Trans-spliced
196 bp product
1 2 3 4 5 6 7 8 9 10

FIG.6B

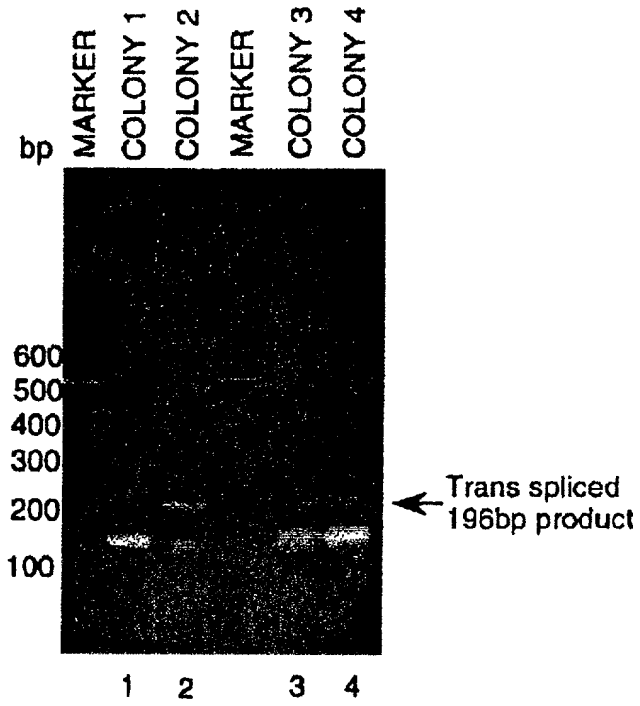

bp
MARKER
COLONY 1
COLONY 2
MARKER
COLONY 3
COLONY 4
600
500
400
300
200
100
1
2
3
4
Trans spliced
196bp product

FIG.7A

EXON 1 OF βHCG6

5'-CAGGGGACGCACCAAGGATGGAGATGTTCCAG-GGCGCTGATGATGTTGTT

1ST CODING NUCLEOTIDE OF DT-A

GATTCTTCTTAAATCTTTTGTGATGGAAAACTTTTCTTCGTACCACGGGACTA

AACCTGGTTATGTAGATTCCATTCAAAAA-3'

FIG.7B

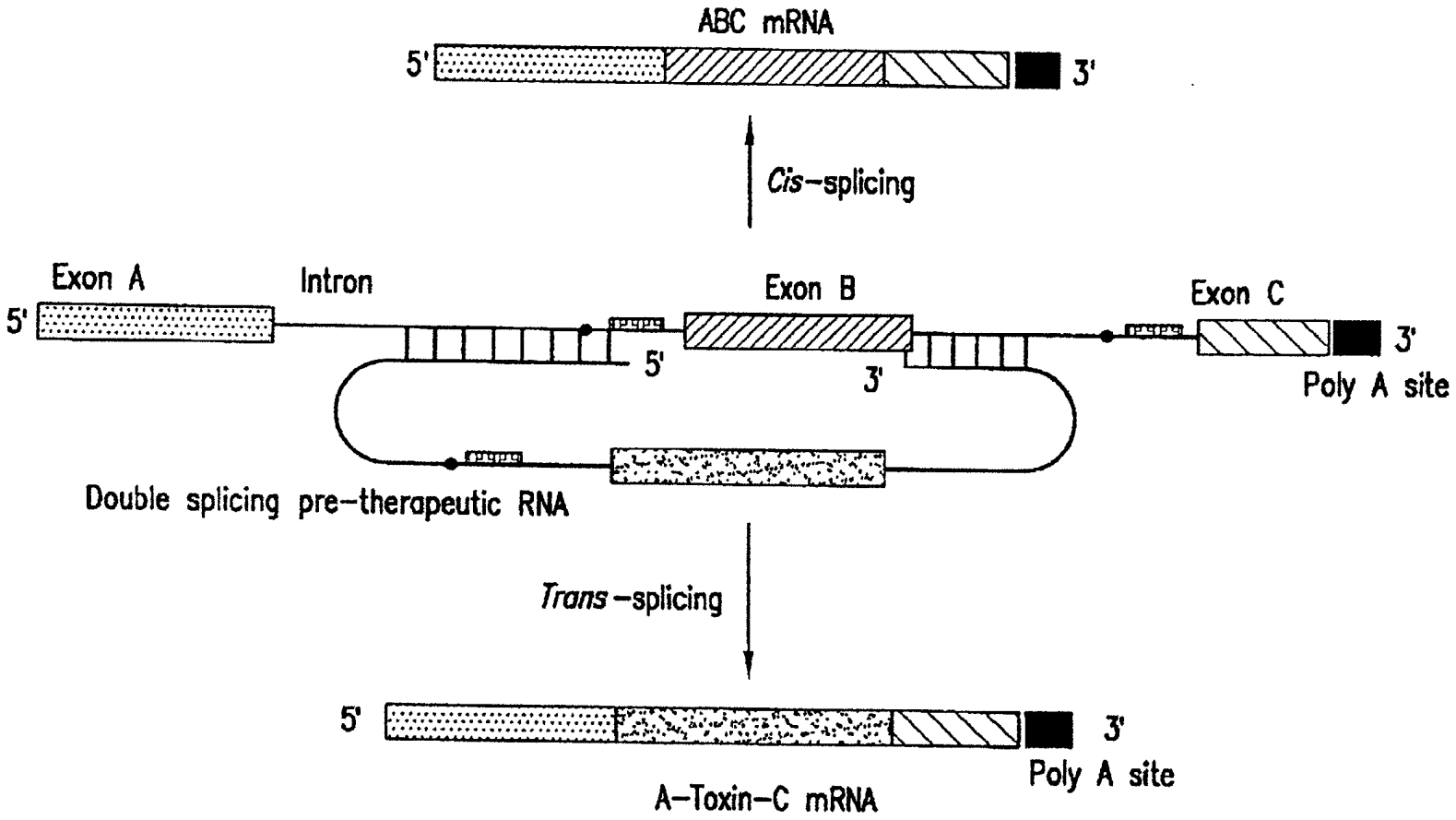
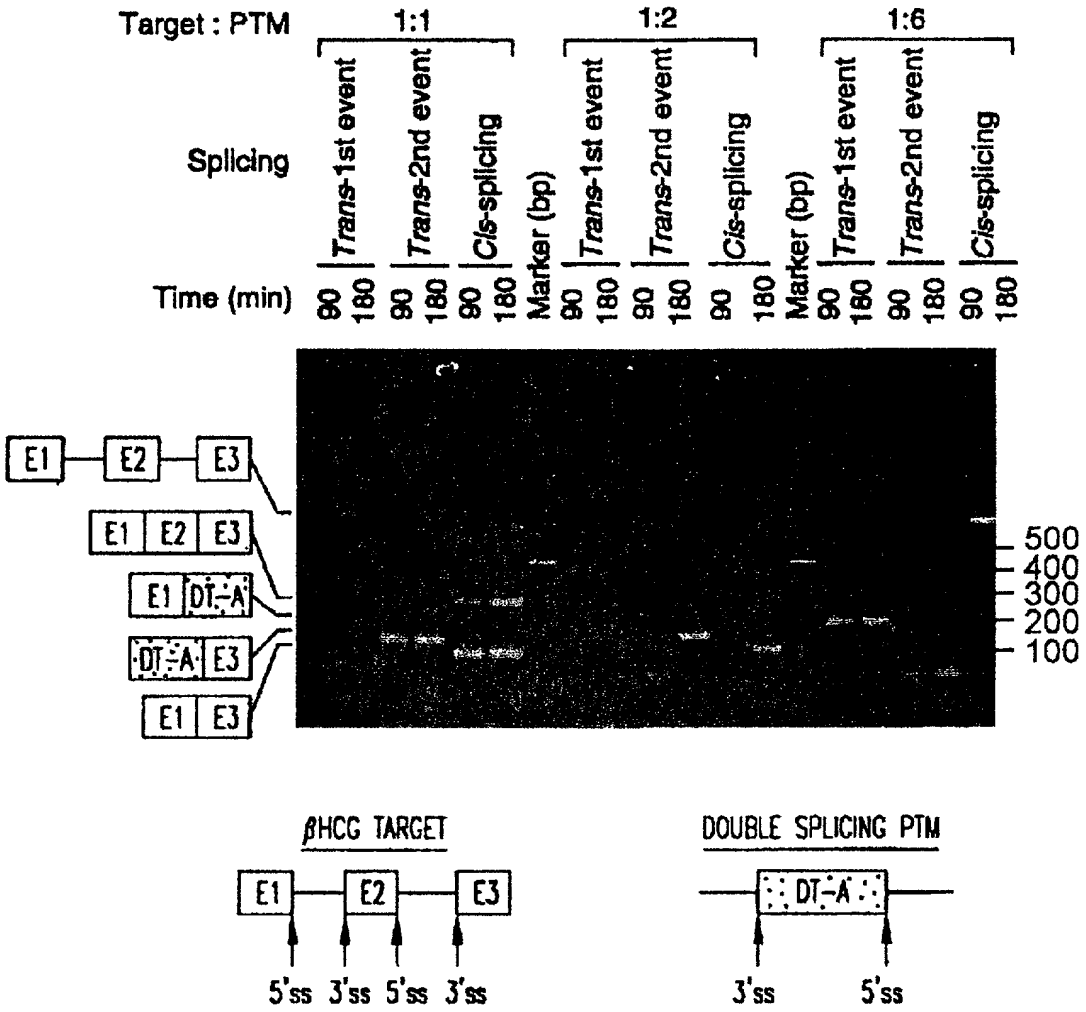


FIG.8A



Cis-spliced products

E1 E2 E3 = NORMAL *cis*-SPLICING (277bp)

E1 E3 = Exon SKIPPING (110bp)

Trans- spliced products

E1 DT-A = 1st EVENT, 196bp. *Trans*-SPLICING BETWEEN 5' ss OF TARGET & 3' ss OF PTM.

DT-A E3 = 2nd EVENT, 161bp. *Trans*- SPLICING BETWEEN 3' ss OF TARGET & 5' ss OF PTM.

FIG.8B

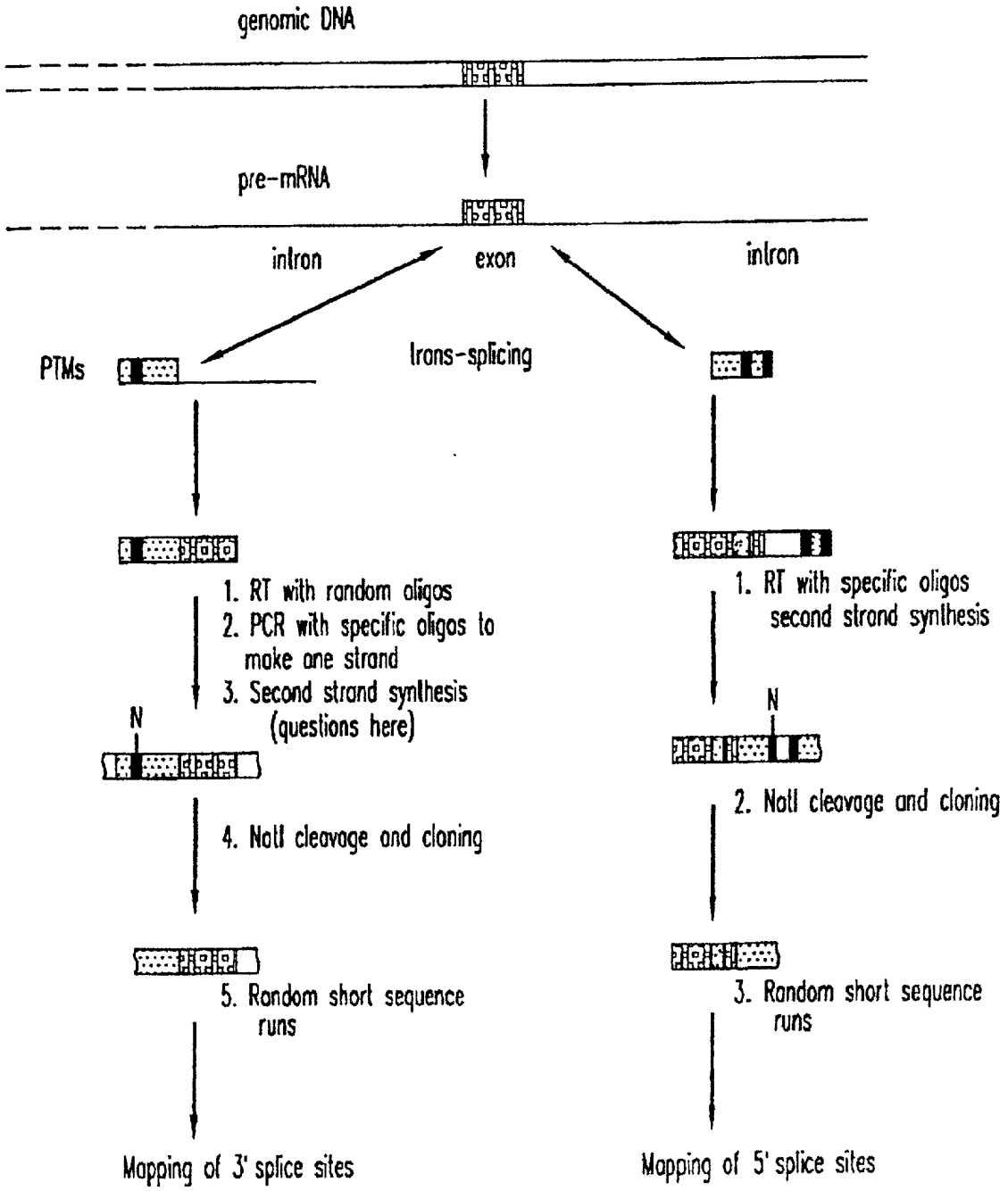

genomic DNA
pre-mRNA
intron
exon
intron
PTMs
Irons-splicing
1. RT with random oligos
2. PCR with specific oligos to make one strand
3. Second strand synthesis (questions here)
N
4. NotI cleavage and cloning
5. Random short sequence runs
Mapping of 3' splice sites
1. RT with specific oligos second strand synthesis
N
2. NotI cleavage and cloning
3. Random short sequence runs
Mapping of 5' splice sites

FIG.9

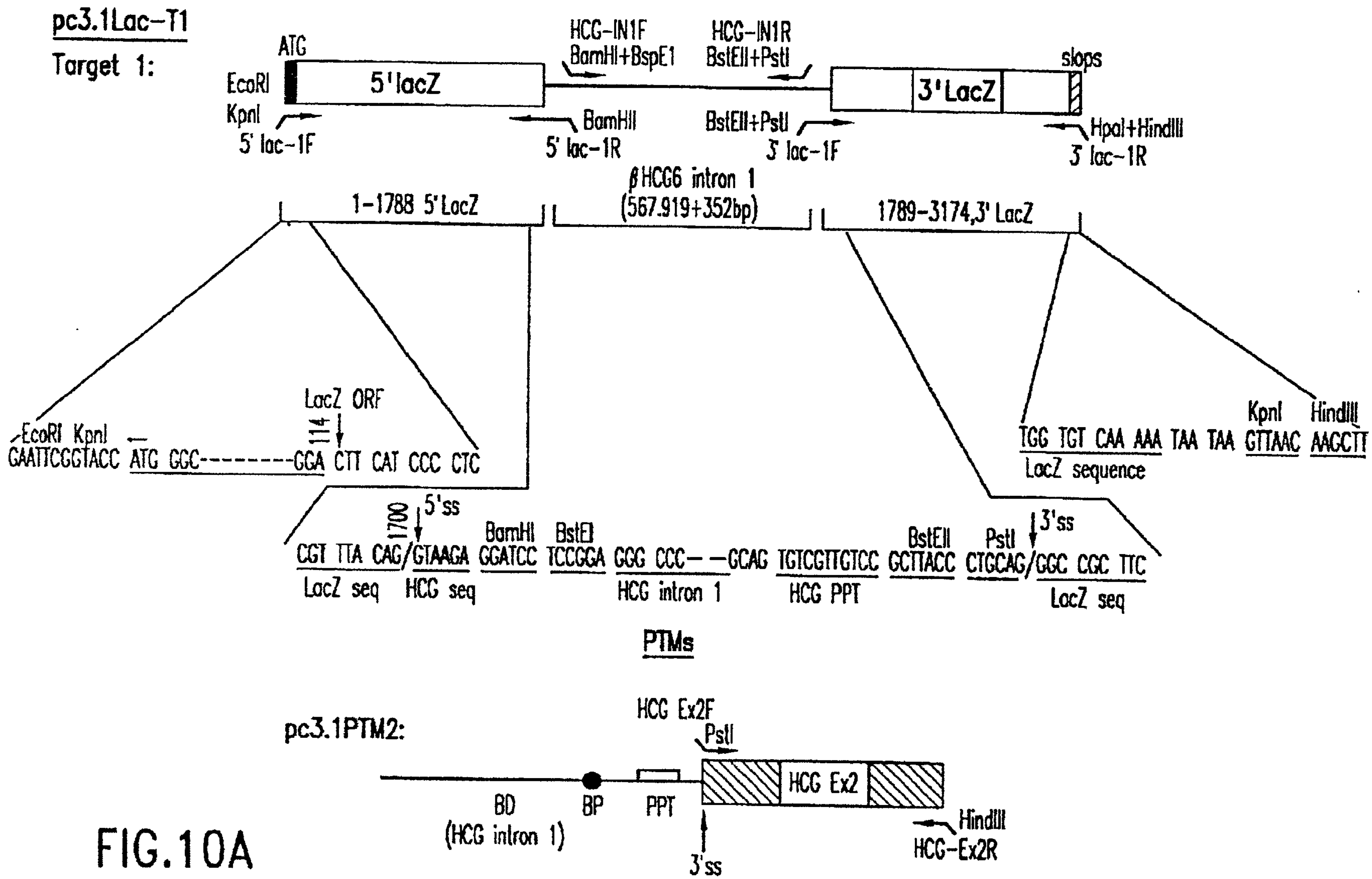

pc3.1Lac-T1
Target 1:
ATG
EcoRI
KpnI
5'lacZ
5' lac-1F
HCG-IN1F
BamHI+BspE1
BamHI
5' lac-1R
HCG-IN1R
BstEII+PstI
BstEII+PstI
3' lac-1F
3'LacZ
stops
HpaI+HindIII
3' lac-1R
1-1788 5'LacZ
βHCG6 intron 1
(567.919+352bp)
1789-3174,3' LacZ
EcoRI KpnI
GAATTCGGTACC ATG GGC--------GGA CTT CAT CCC CTC
LacZ ORF
114
TGG TGT CAA AAA TAA TAA GTTAAC AAGCTT
KpnI
HindIII
LacZ sequence
1700
5'ss
BamHI
BstEI
BstEII
PstI
3'ss
CGT TTA CAG/GTAAGA GGATCC TCCGGA GGG CCC--GCAG TGTCGTTGTCC GCTTACC CTGCAG/GGC CGC TTC
LacZ seq
HCG seq
HCG intron 1
HCG PPT
LacZ seq
PTMs
pc3.1PTM2:
HCG Ex2F
PstI
HCG Ex2
BD
(HCG intron 1)
BP
PPT
3'ss
HindIII
HCG-Ex2R

FIG.10A

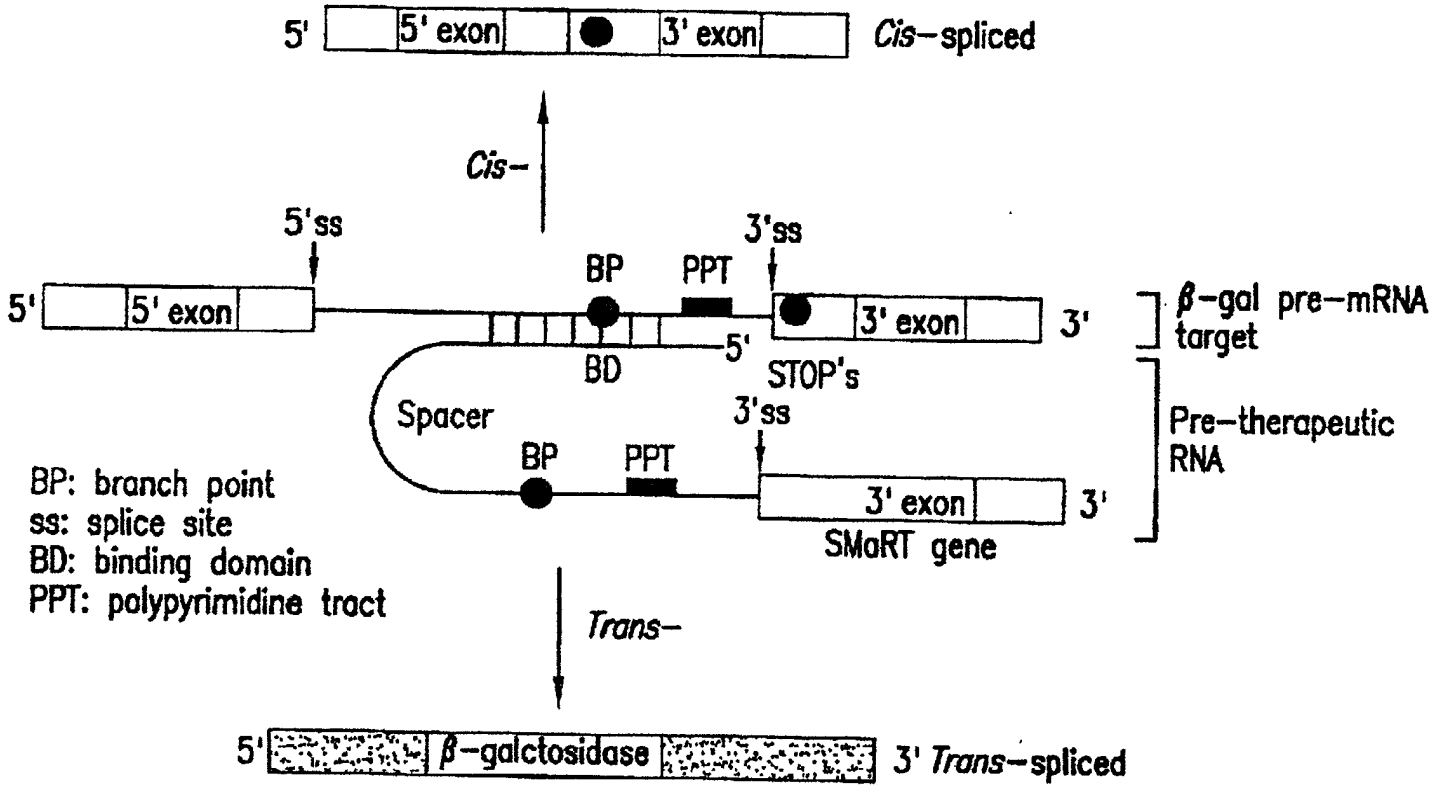

5'
5' exon
3' exon
Cis–spliced
Cis–
5'ss
3'ss
BP
PPT
5'
5' exon
3' exon
3'
β–gal pre–mRNA target
BD
5'
STOP's
Spacer
3'ss
Pre–therapeutic RNA
BP
PPT
3' exon
3'
SMaRT gene
BP: branch point
ss: splice site
BD: binding domain
PPT: polypyrimidine tract
Trans–
5'
β–galctosidase
3' Trans–spliced

FIG.10B

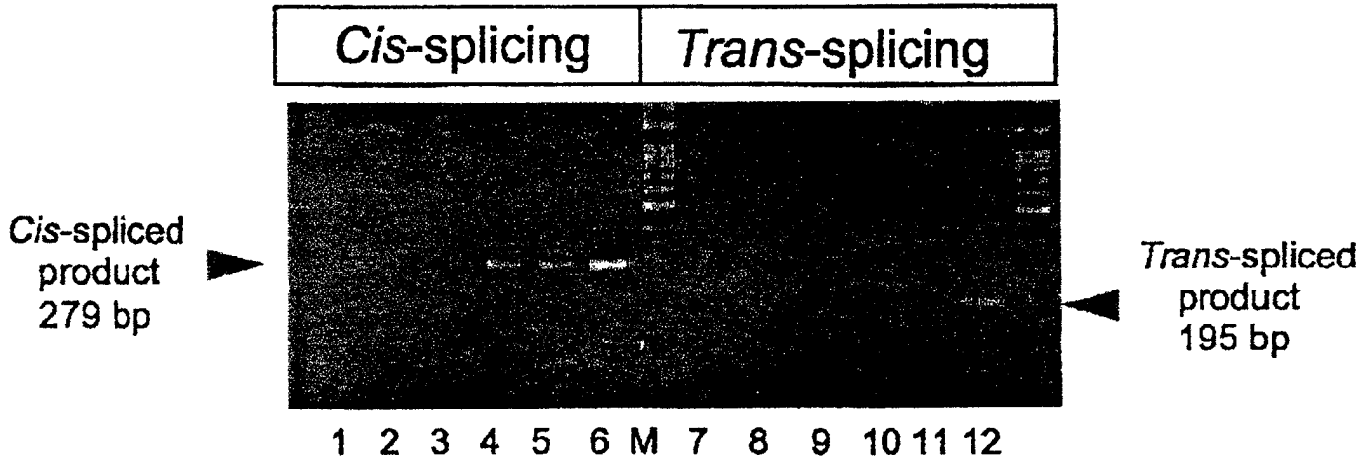


FIG.11A

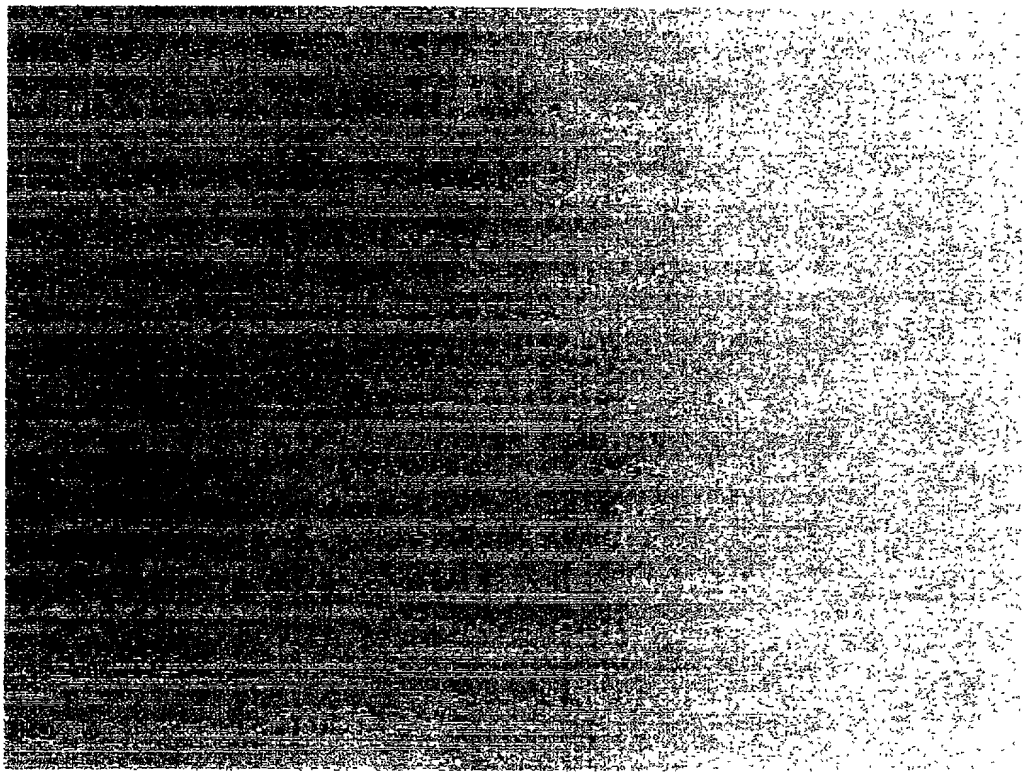


FIG.11B

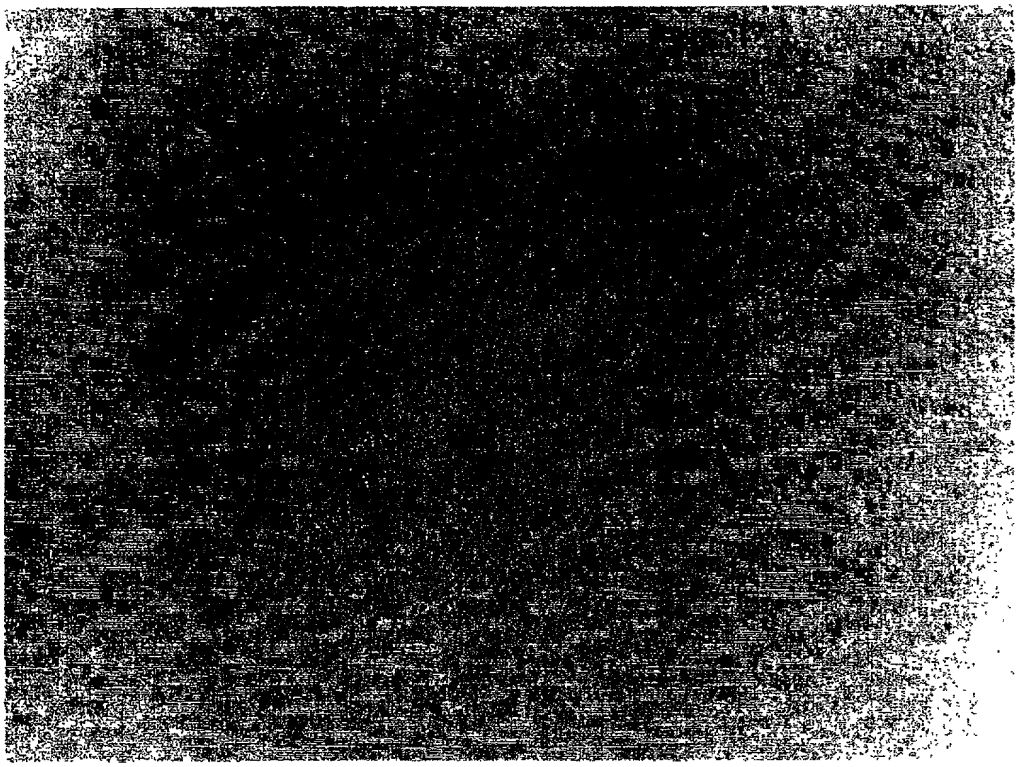


FIG.11C

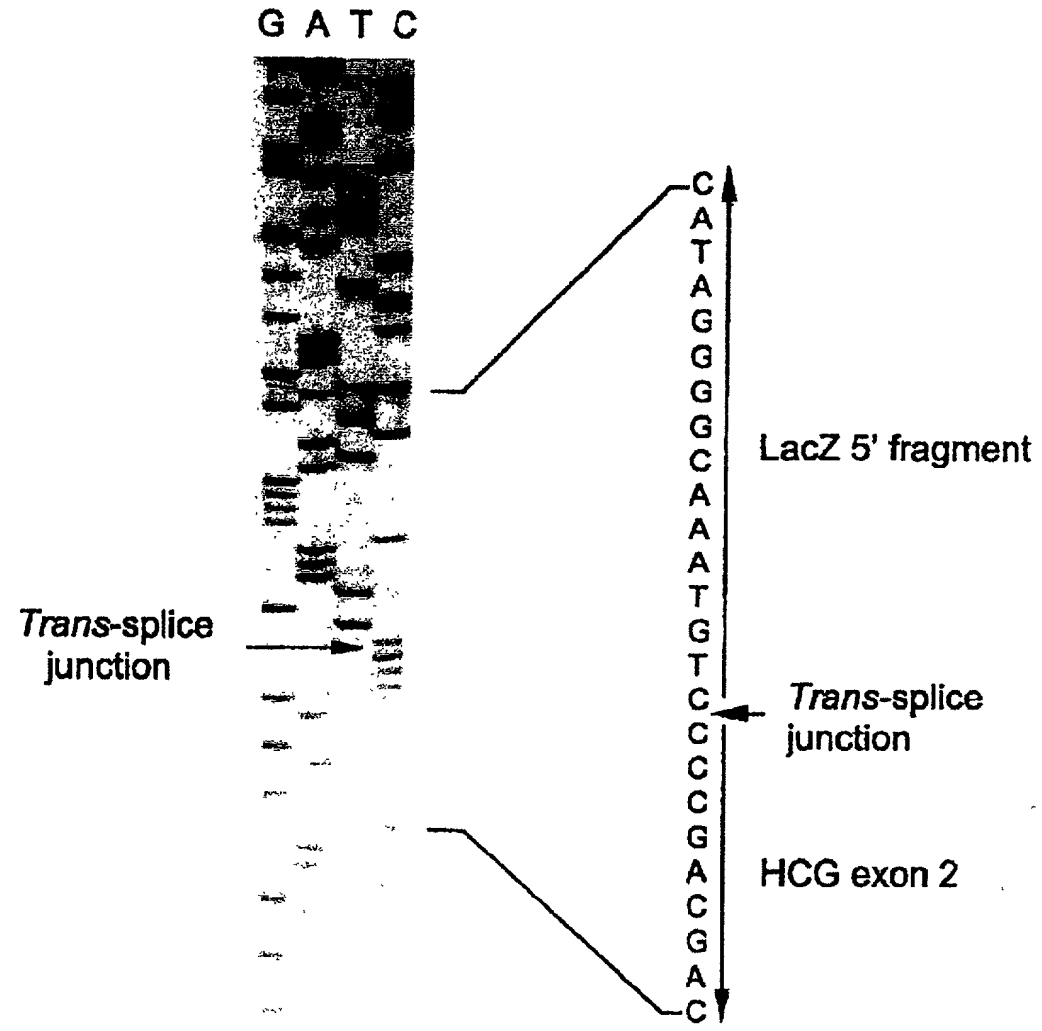

G A T C
C A T A G G G G C A A A T G T C C C C G A C G A C
LacZ 5' fragment
Trans-splice junction
Trans-splice junction
HCG exon 2

FIG.12A

1. NUCLEOTIDE SEQUENCES OF THE *cis*-SPLICED PRODUCT (285 bp):

BioLac-TR1

GGCTTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCCACGCGATGGGTAACAGTCTTG

Splice junction

GCGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAGTATCCCCGTTTACAG/GGCGGCTTCGTCTAATAATG

GGACTGGGTGGATCAGTCGCTGATTAAATATGATGAAAACGGCAACCCGTGGTCGGCTTACGGCGGTGATTT

Lac-TR2

TGGCGATACGCCGAACGATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCAG

2. NUCLEOTIDE SEQUENCES OF THE *trans*-SPLICED PRODUCT (195 bp)

BioLac-TR1

GGCTTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCCACGCGATGGGTAACAGTCTTGG

Splice junction

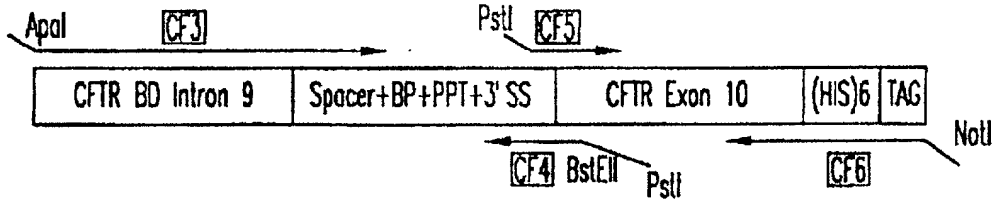
CGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAGTATCCCCGTTTACAG/GGGCTGCTGCTGTTGCTGCTGCT

HCGR2

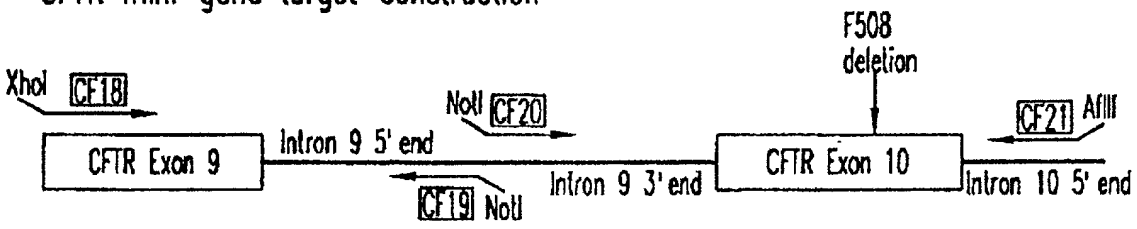
GAGCATGGGCGGGACATGGGCATCCAAGGAGCCACTTCGGCCACGGTGCCG

FIG.12B

CFTR Pre-therapeutic molecule (PTM or "bullet")



CFTR mini-gene target-construction



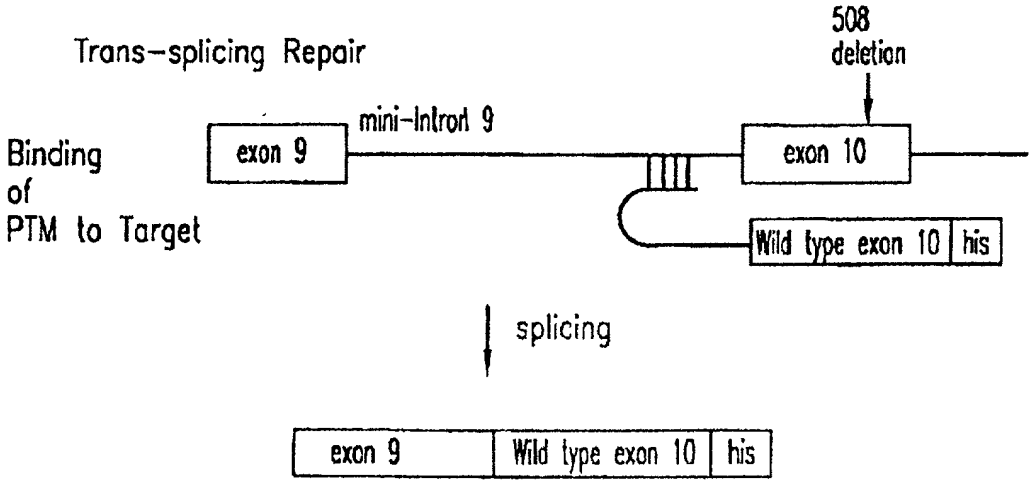


FIG.13

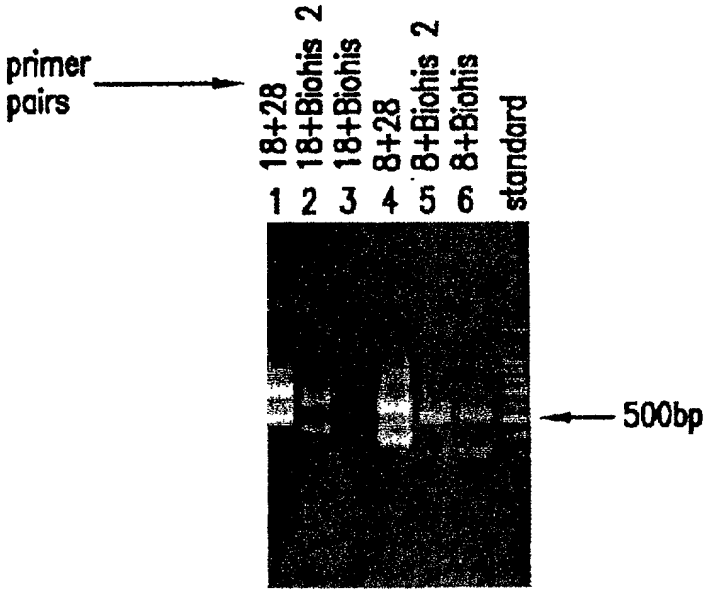

primer pairs
18+28
18+Biohis 2
18+Biohis
8+28
8+Biohis 2
8+Biohis
standard
1 2 3 4 5 6
500bp

FIG.14

DNA sequence 500 b.p. GCTAGCGTTTAA ... TGCCACTCCCAC linear

Positions of Restriction Endonucleases sites (unique sites underlined)

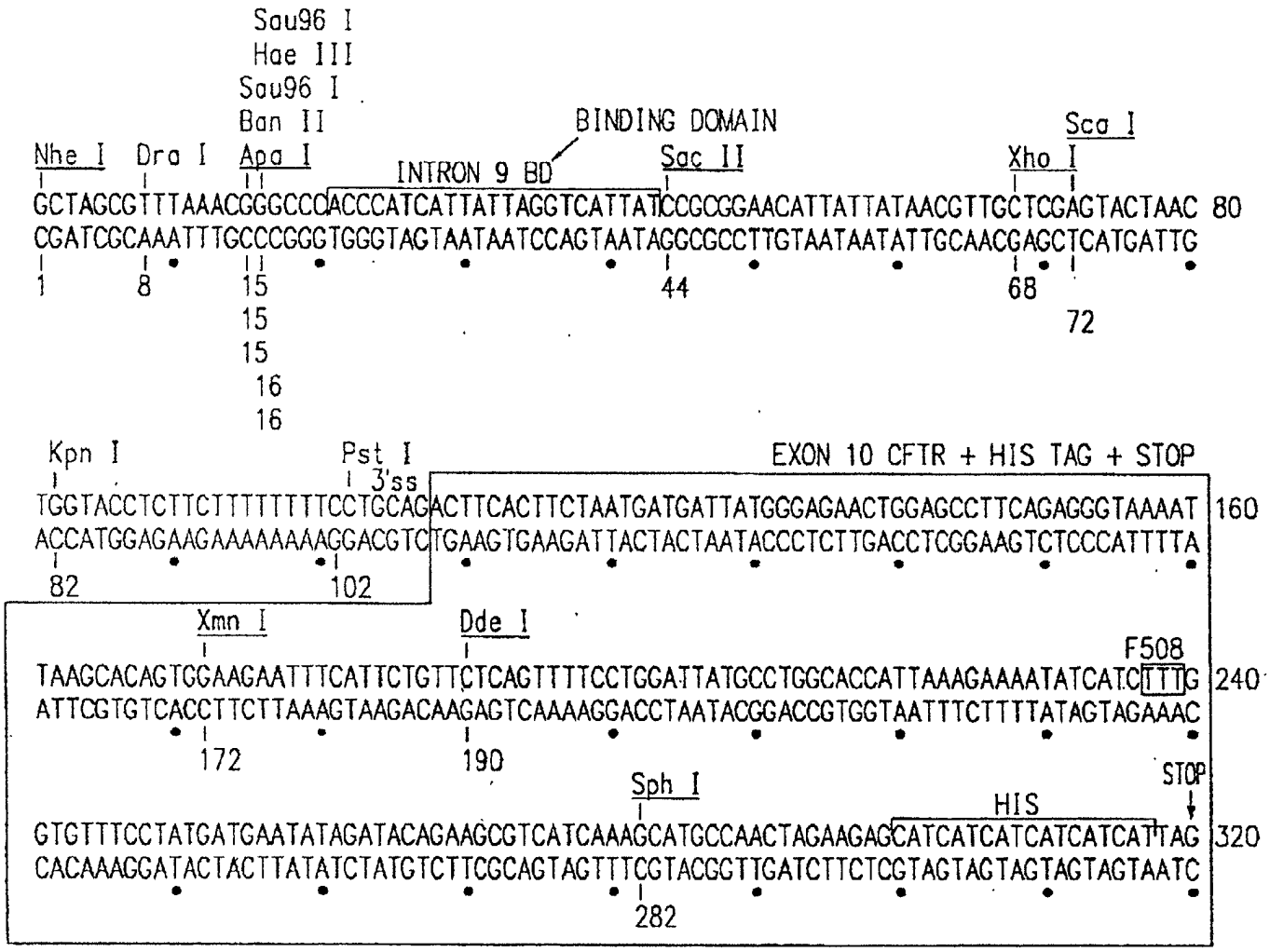
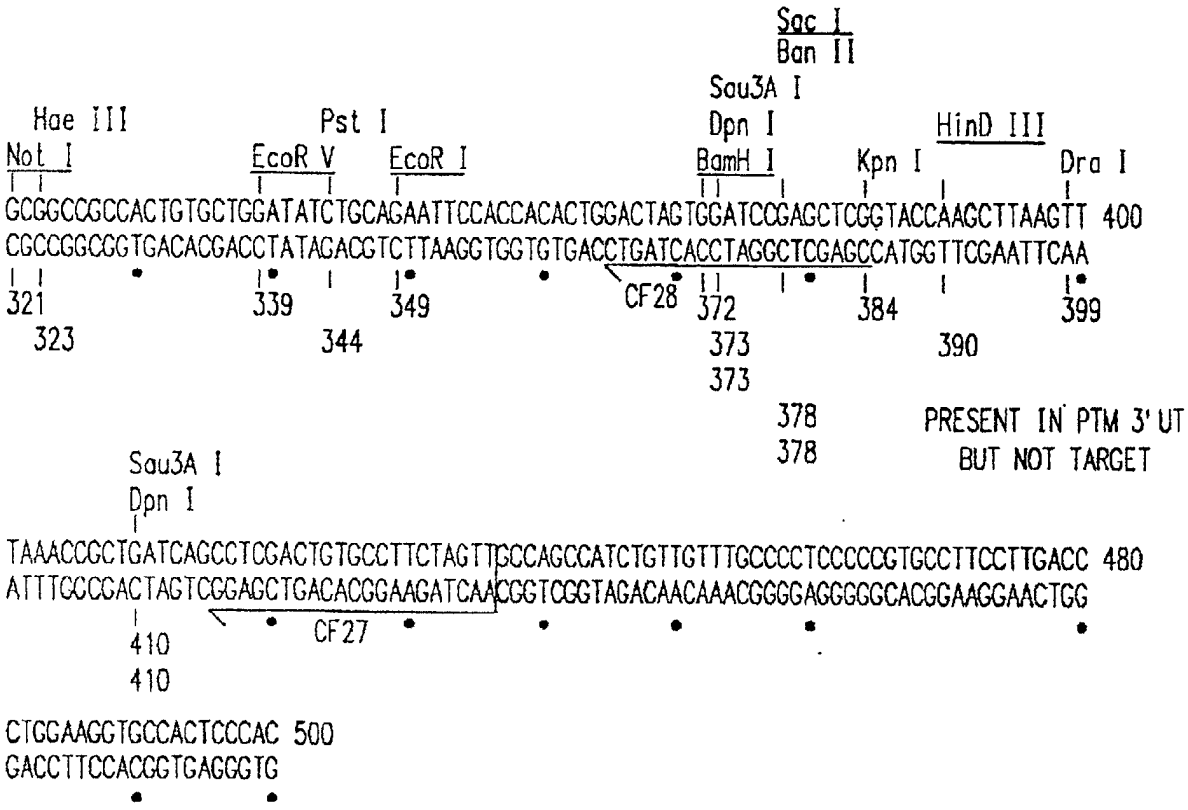


FIG.15A



Restriction Endonucleases site usage

Acc I	–	EcoR I	1	Nde I	–	Sau96 I	2
Apa I	1	EcoR V	1	Nhe I	1	Sca I	1
ApaL I	–	Hae II	–	Not I	1	Sma I	–
Avr II	–	Hae III	2	PflM I	–	Sph I	1
BamH I	1	HinC II	–	Pst I	2	Spl I	–
Ban II	2	HinD III	1	Pvu I	–	Ssp I	–
Bbe I	–	Hinf I	–	Pvu II	–	Stu I	–

FIG.15B

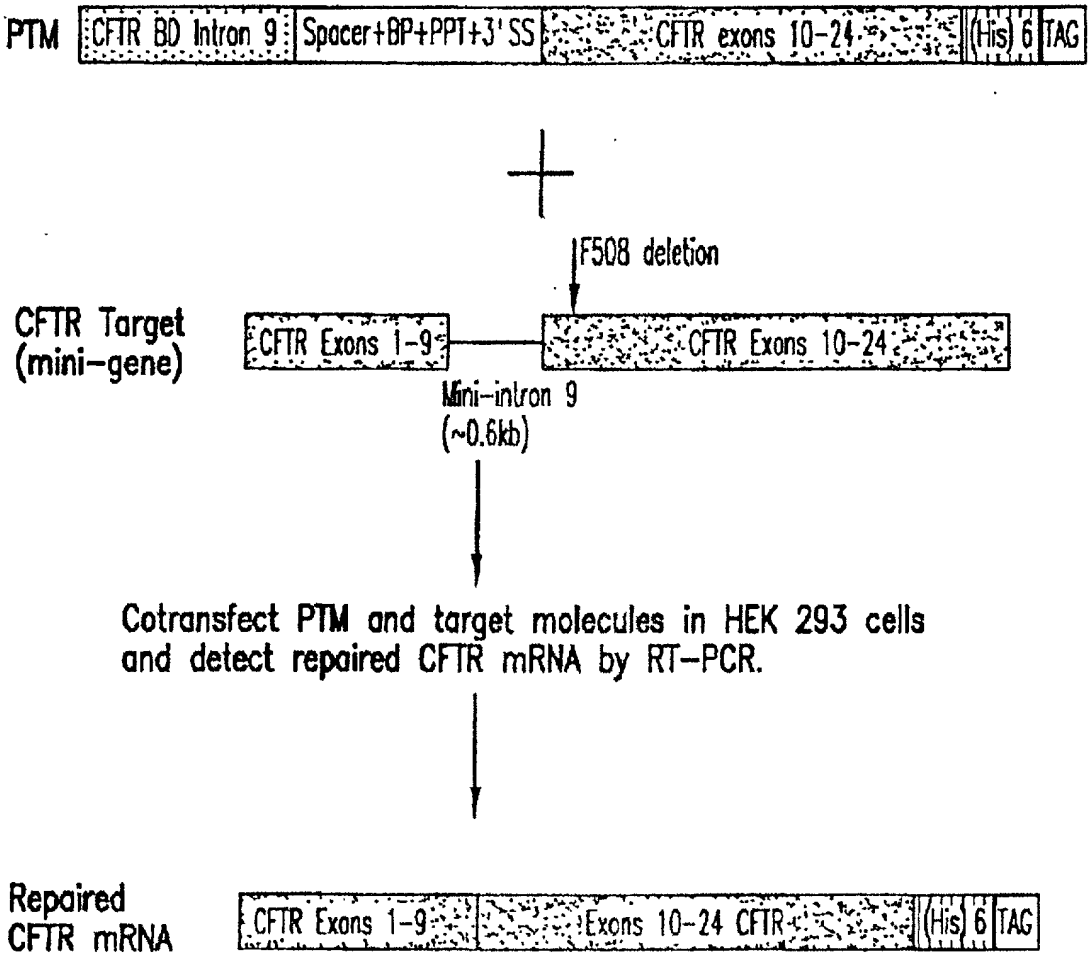

PTM
CFTR BD Intron 9
Spacer+BP+PPT+3' SS
CFTR exons 10-24
(His) 6
TAG
F508 deletion
CFTR Target
(mini-gene)
CFTR Exons 1-9
CFTR Exons 10-24
Mini-intron 9
(~0.6kb)
Cotransfect PTM and target molecules in HEK 293 cells
and detect repaired CFTR mRNA by RT-PCR.
Repaired
CFTR mRNA
CFTR Exons 1-9
Exons 10-24 CFTR
(His) 6
TAG

FIG.16

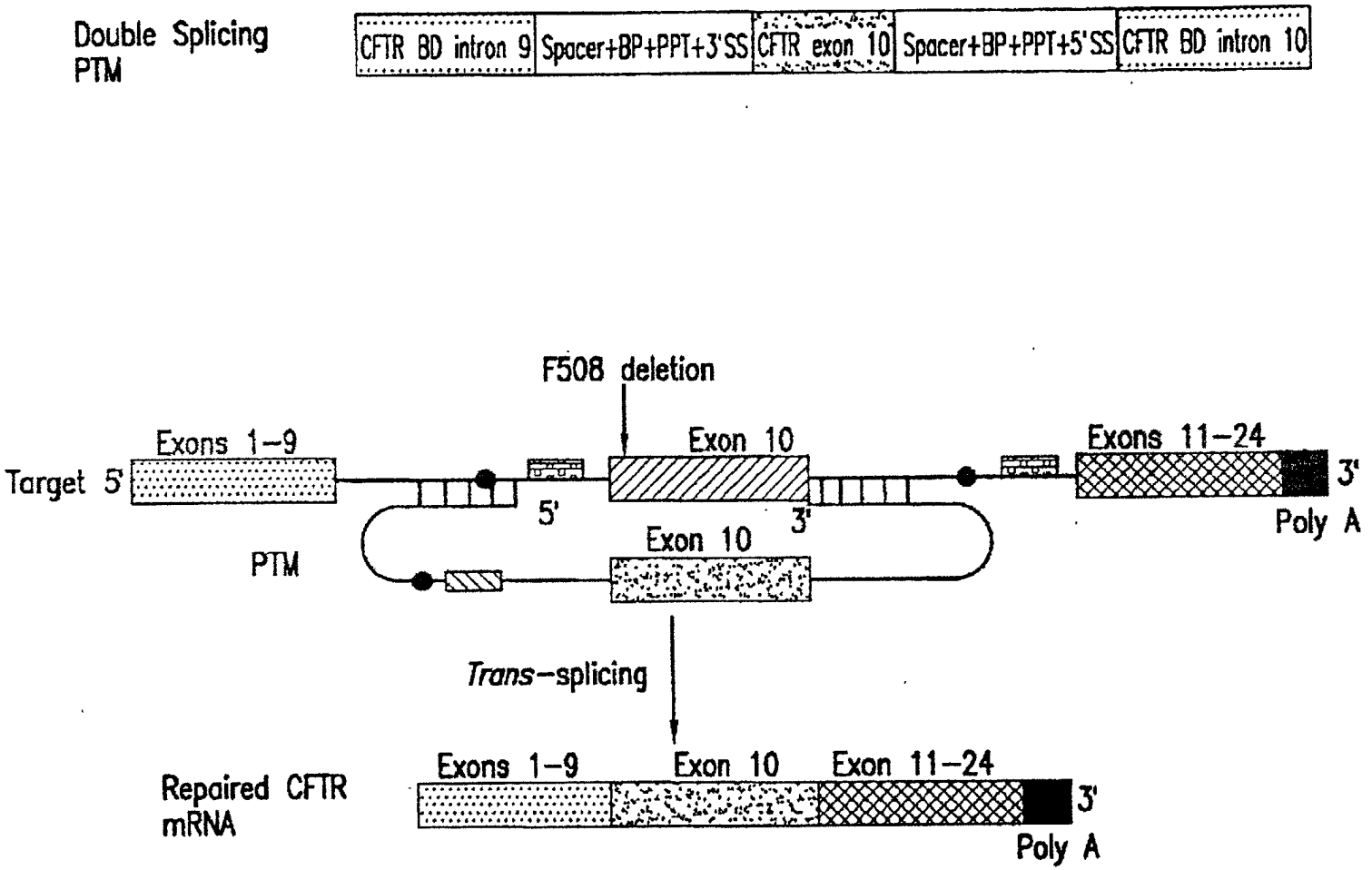

Double Splicing PTM
CFTR BD intron 9
Spacer+BP+PPT+3'SS
CFTR exon 10
Spacer+BP+PPT+5'SS
CFTR BD intron 10
F508 deletion
Exons 1–9
Exon 10
Exons 11–24
Target 5'
3'
5'
3'
Poly A
PTM
Exon 10
Trans–splicing
Repaired CFTR mRNA
Exons 1–9
Exon 10
Exon 11–24
3'
Poly A

FIG.17

DOUBLE *TRANS*-SPLICING SPECIFIC TARGET

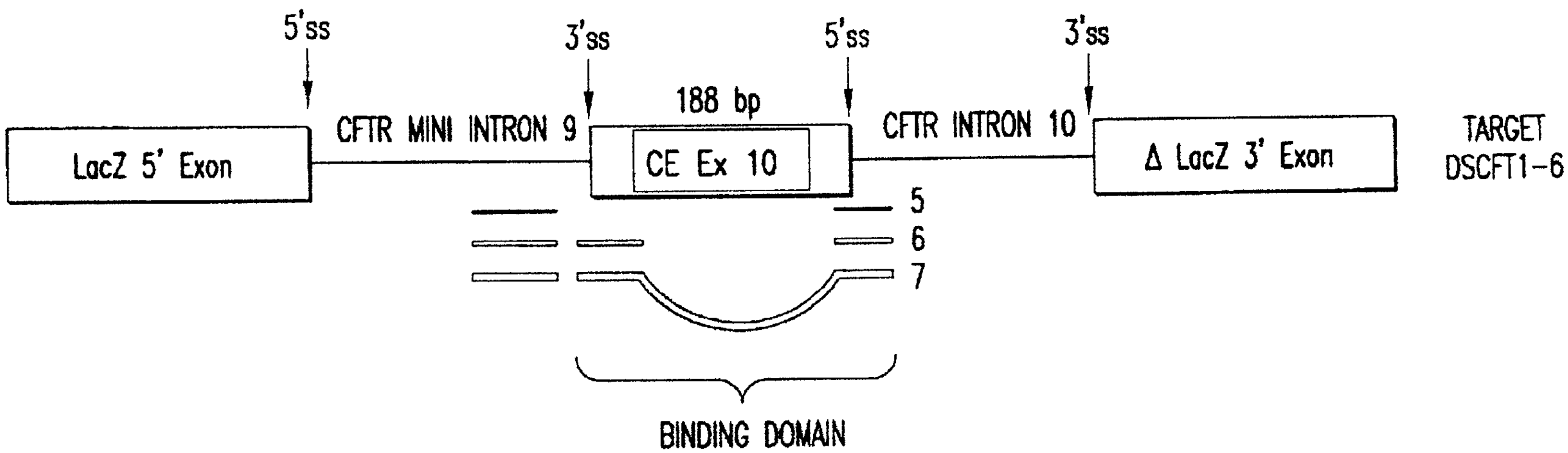


FIG.18

DOUBLE *TRANS*-SPLICING PTMs

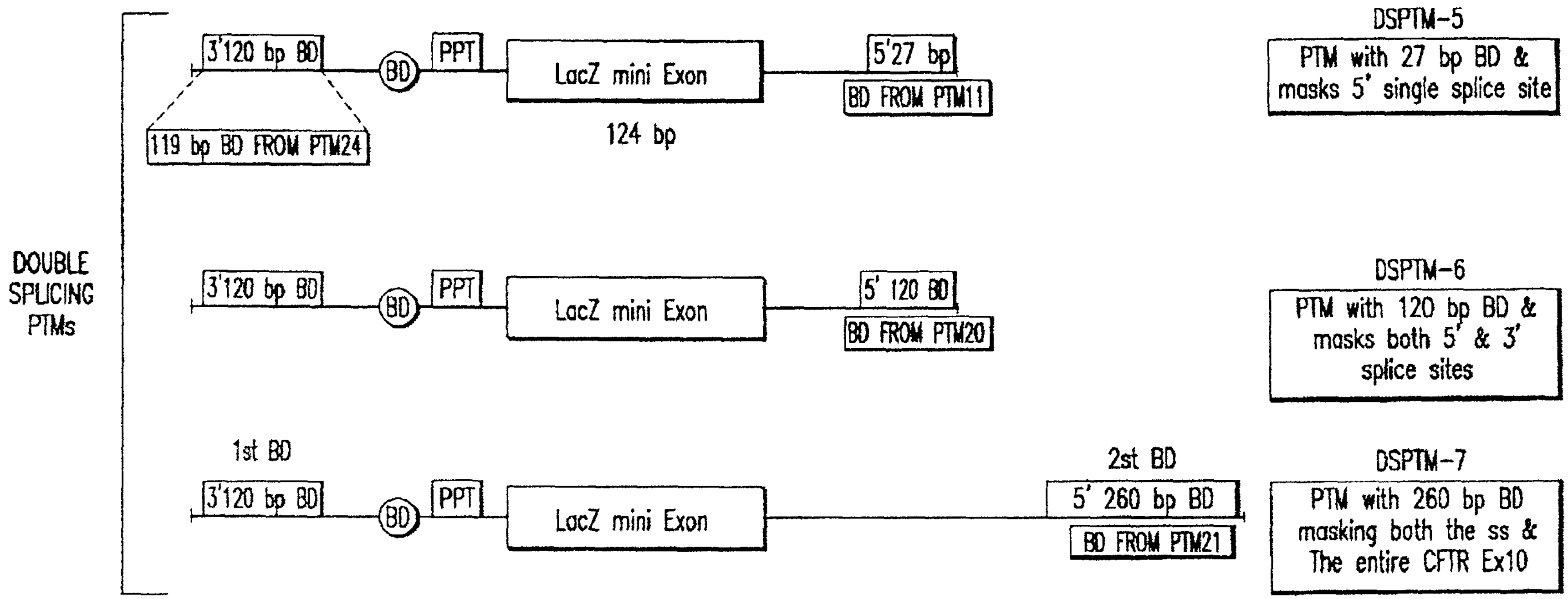


FIG.19

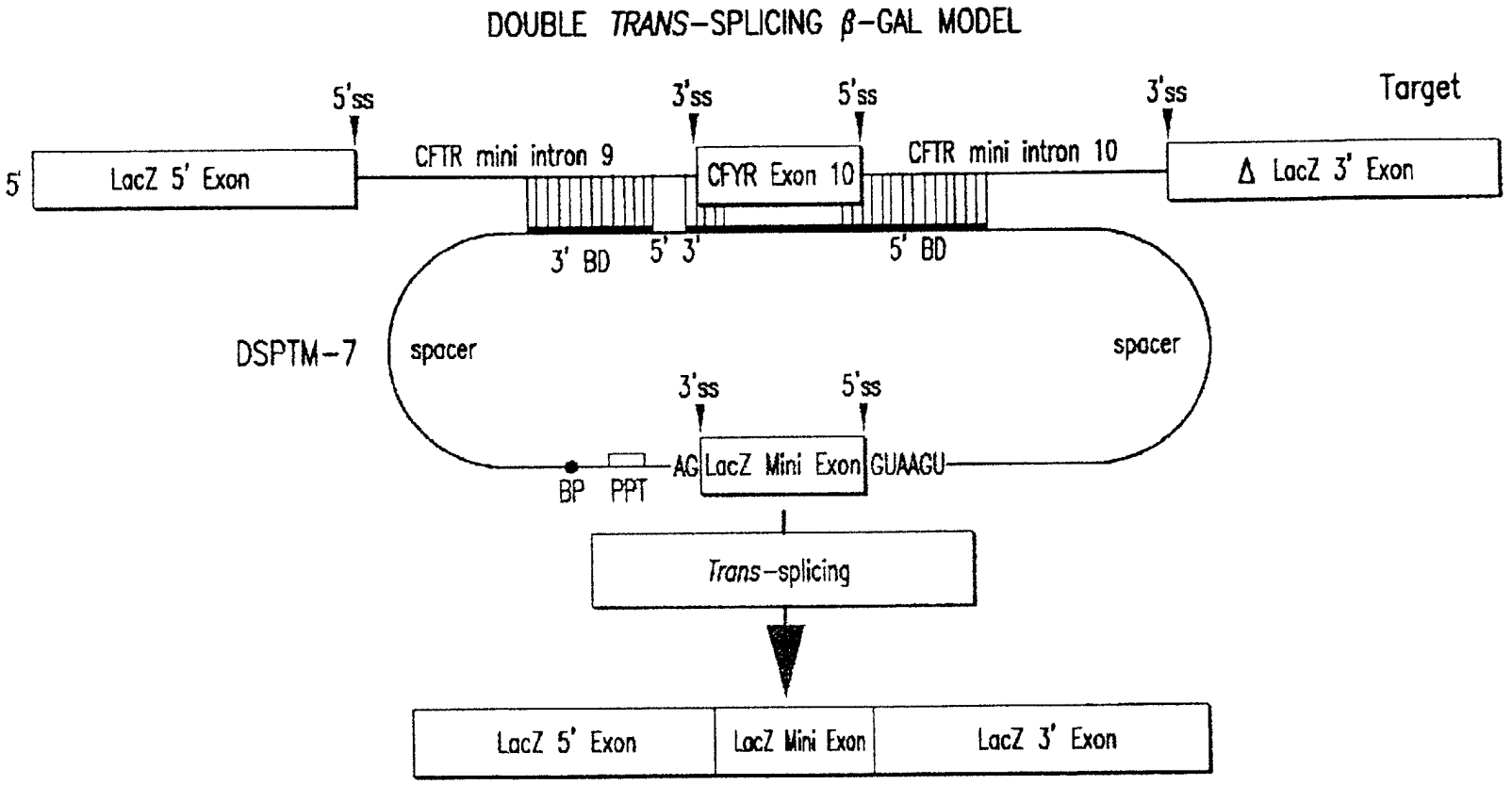
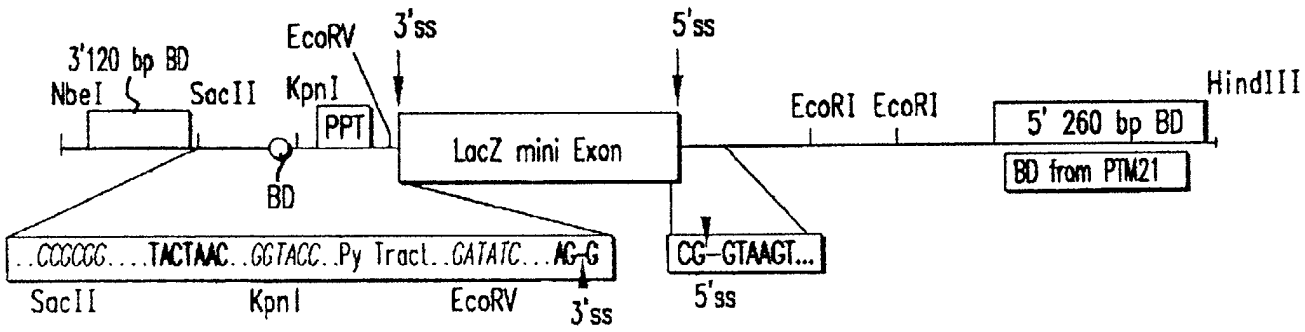

DOUBLE TRANS-SPLICING β-GAL MODEL
5'ss
3'ss
5'ss
3'ss
Target
5'
LacZ 5' Exon
CFTR mini intron 9
CFYR Exon 10
CFTR mini intron 10
Δ LacZ 3' Exon
3' BD
5' 3'
5' BD
DSPTM-7
spacer
spacer
3'ss
5'ss
BP
PPT
AG
LacZ Mini Exon
GUAAGU
Trans-splicing
LacZ 5' Exon
LacZ Mini Exon
LacZ 3' Exon
Repaired LacZ mRNA

FIG.20



(1) 3' BD (120 BP): GATTCACTTGCTCCAATTATCATCCTAAGCAGAACTGTATATTCTTATTTGTAAAGATTCTATTAACTCATTTGATTC
AAAATATTTAAAATACTTCCTGTTTCATACTCTGCTATGCAC

(2) Spacer sequences (24 bp): AACATTATTATAACGTTGCTCGAA

(3) Branch point, pyrimidine tract and acceptor splice site: TACTAAC T *GGTACC* TCTTCTTTTTTTTTT *GATATC* CTGCAG GGC GGC
BP Kpn I PPT EcoRV 3'ss LacZ mini exon

(4) 5' donor site and 2nd spacer sequence: TGA ACG *GTAAGT* GTTATCACCGATATGTGTCTAACCTGATTCGGGCCTTCGATACG
LacZ mini exon 5'ss
CTAAGATCCACCGG

(5) 5' BD (260 BP): TCAAAAAGTTTTCACATAATTTCTTACCTCTTCTT*GAATTC*ATGCTTTGATGACGCTTCTGTATCTATATTCATCATTGGAA
ACACCAATGATTTTTCTTTAATGGTGCCTGGCATAATCCTGGAAAACTGATAACACAATGAAATTCTTCCACTGTGCTTAA
AAAAACCCTCT*GAATTC*TCCATTTCTCCCATAATCATCATTACAACTGAACTCTGGAAATAAAACCCATCATTATTAACTCA
TTATCAAATCACGC

FIG.21

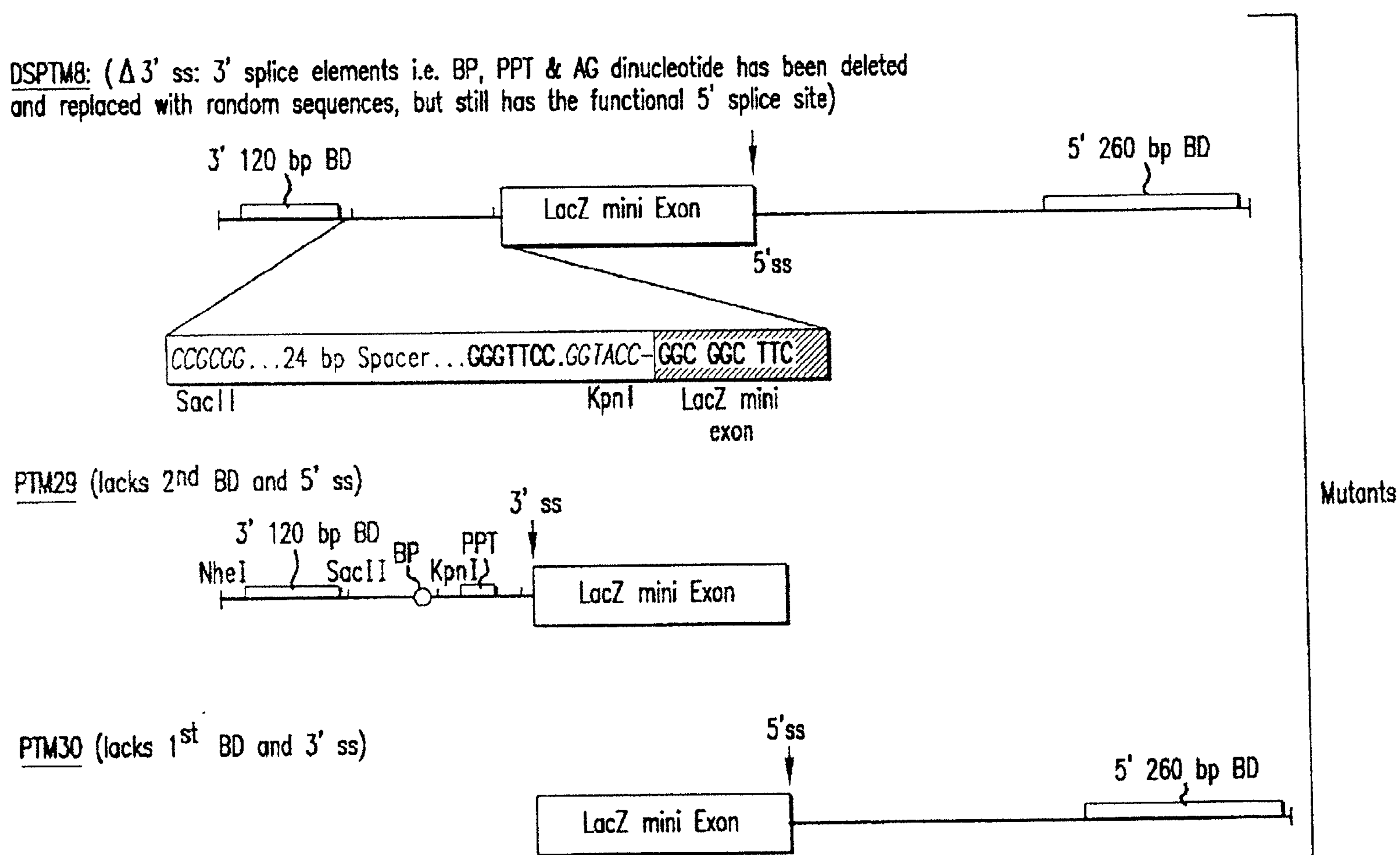

DSPTM8: (Δ 3' ss: 3' splice elements i.e. BP, PPT & AG dinucleotide has been deleted and replaced with random sequences, but still has the functional 5' splice site)
3' 120 bp BD
LacZ mini Exon
5'ss
5' 260 bp BD
CCGCGG...24 bp Spacer...GGGTTCC.GGTACC-GGC GGC TTC
SacII
KpnI
LacZ mini exon
PTM29 (lacks 2nd BD and 5' ss)
3' 120 bp BD
NheI
SacII
BP
PPT
KpnI
3' ss
LacZ mini Exon
PTM30 (lacks 1st BD and 3' ss)
5'ss
LacZ mini Exon
5' 260 bp BD
Mutants

FIG.22

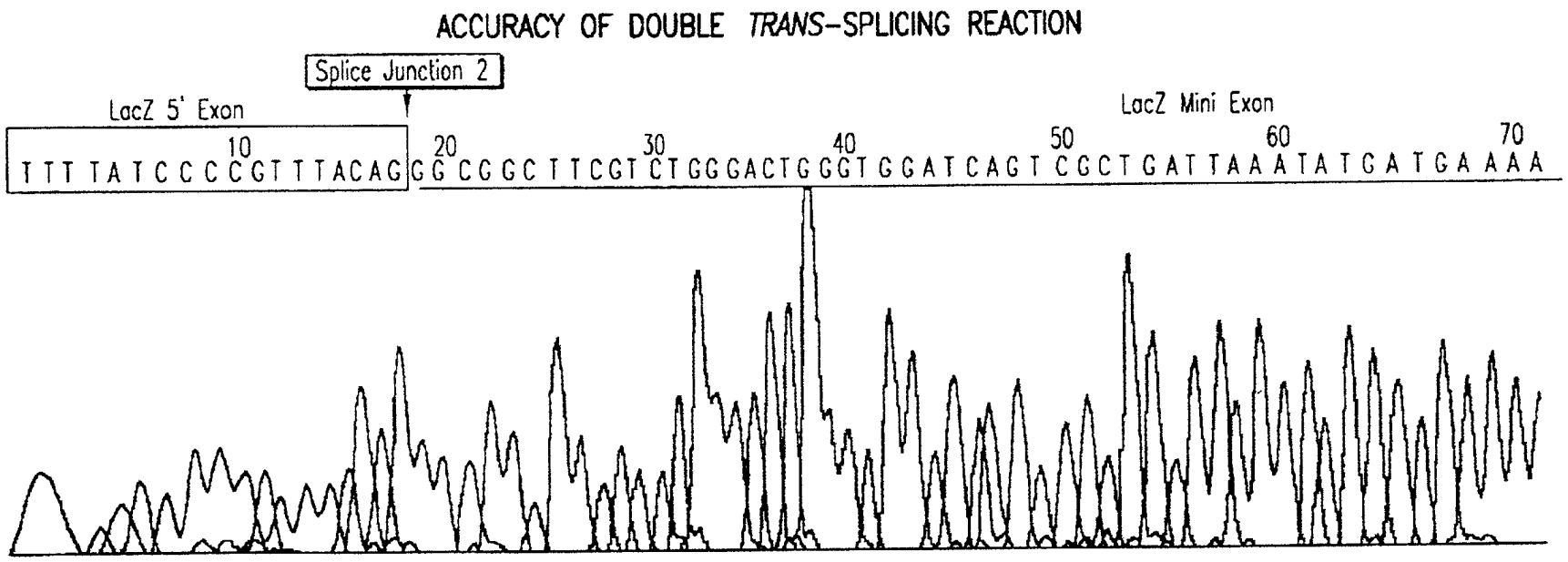

ACCURACY OF DOUBLE TRANS-SPLICING REACTION
Splice Junction 2
LacZ 5' Exon
LacZ Mini Exon
10
20
30
40
50
60
70
TTT TATCCCCGTTTACAG GGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGATTAAATATGATGAAAA

FIG.23A

ACCURACY OF DOUBLE *TRANS*-SPLICING REACTION

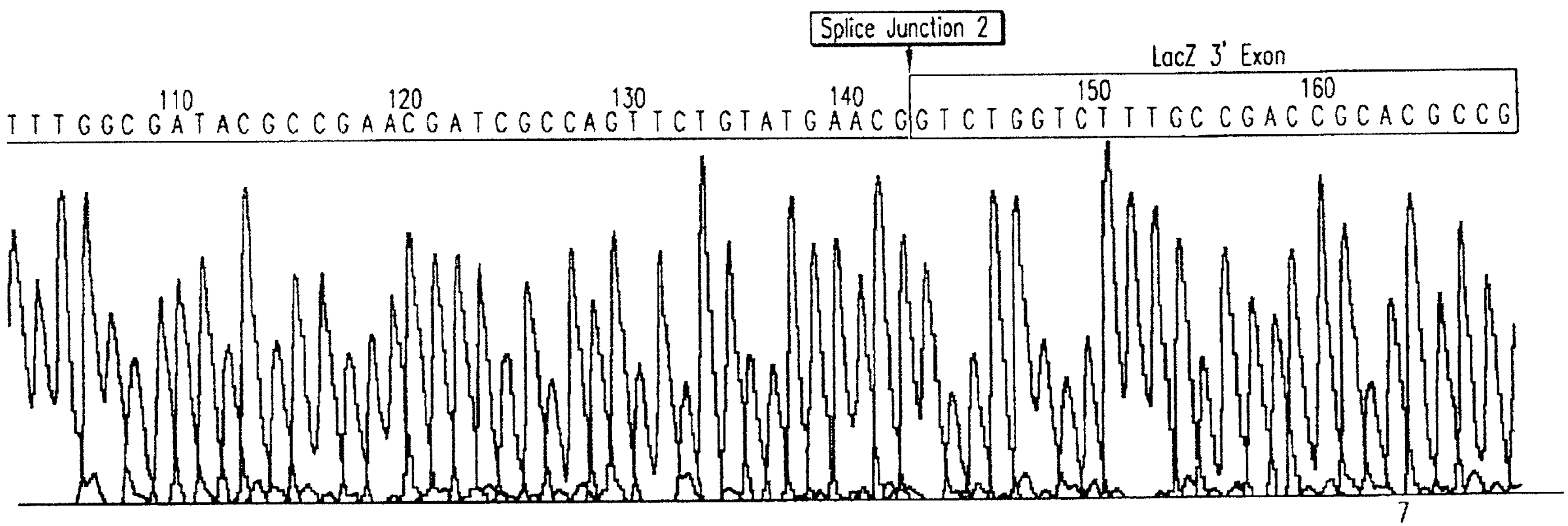


FIG.23B

Double *Trans*-splicing Produces Full-length Protein

Lane 1: DSCFT1.6 Target alone	25 µg
Lane 2: DSPTM7	25 µg
Lane 3 Target + PTM #6	25 µg
Lane 4: Target + PTM #9	25 µg
Lane 5: Delta 3' splice mutant alone	25 µg
Lane 6: Target + Delta 3' ss	25 µg
Lane 7: Target+PTM29+30 (mutants)	25 µg

Figure 24

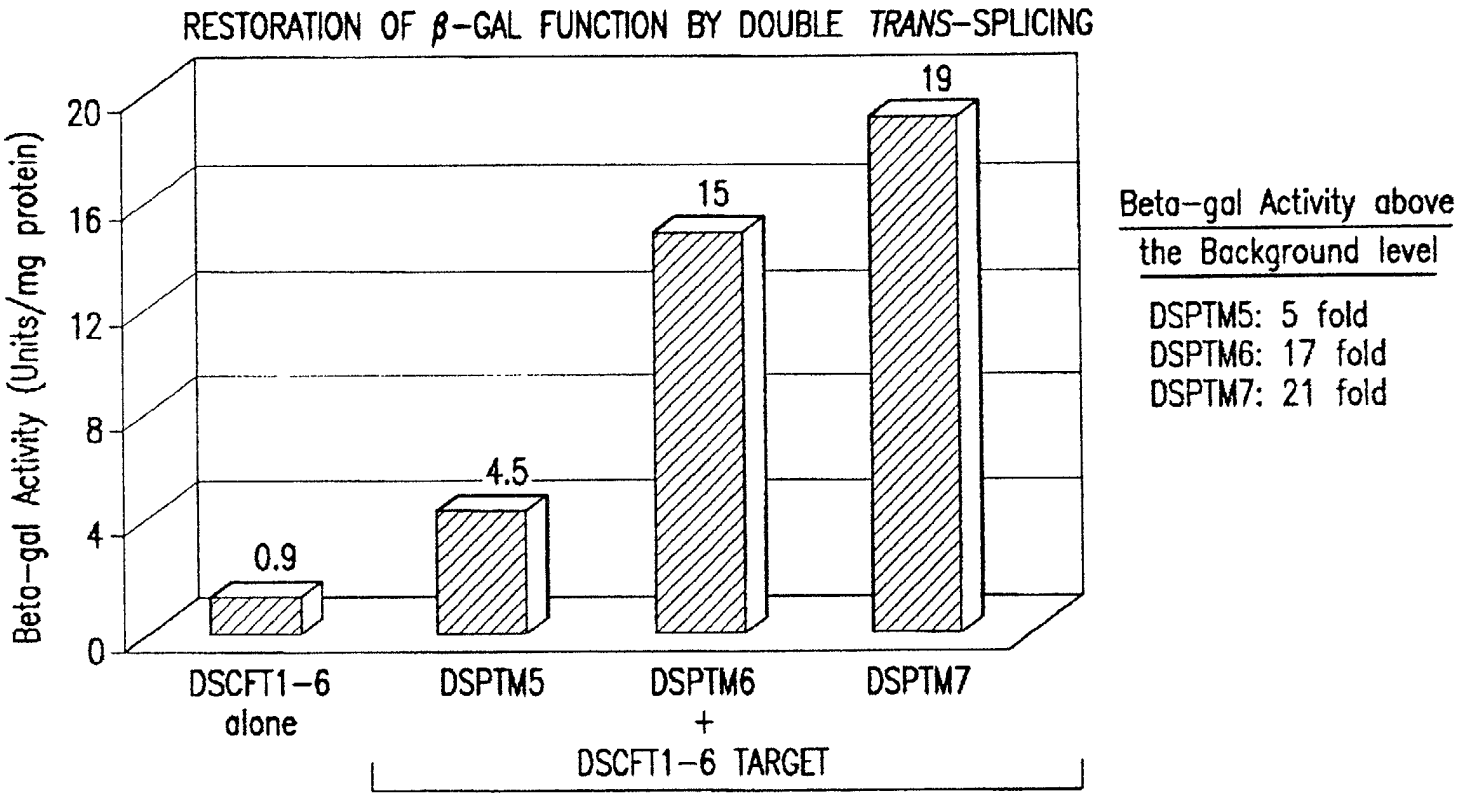

RESTORATION OF β-GAL FUNCTION BY DOUBLE TRANS-SPLICING
Beta-gal Activity (Units/mg protein)
0
4
8
12
16
20
0.9
4.5
15
19
DSCFT1-6 alone
DSPTM5
DSPTM6 + DSCFT1-6 TARGET
DSPTM7
Beta-gal Activity above the Background level
DSPTM5: 5 fold
DSPTM6: 17 fold
DSPTM7: 21 fold

FIG.25

RESTORATION OF β-GAL ACTIVITY IS DUE TO DOUBLE RNA TRANS-SPLICING EVENTS

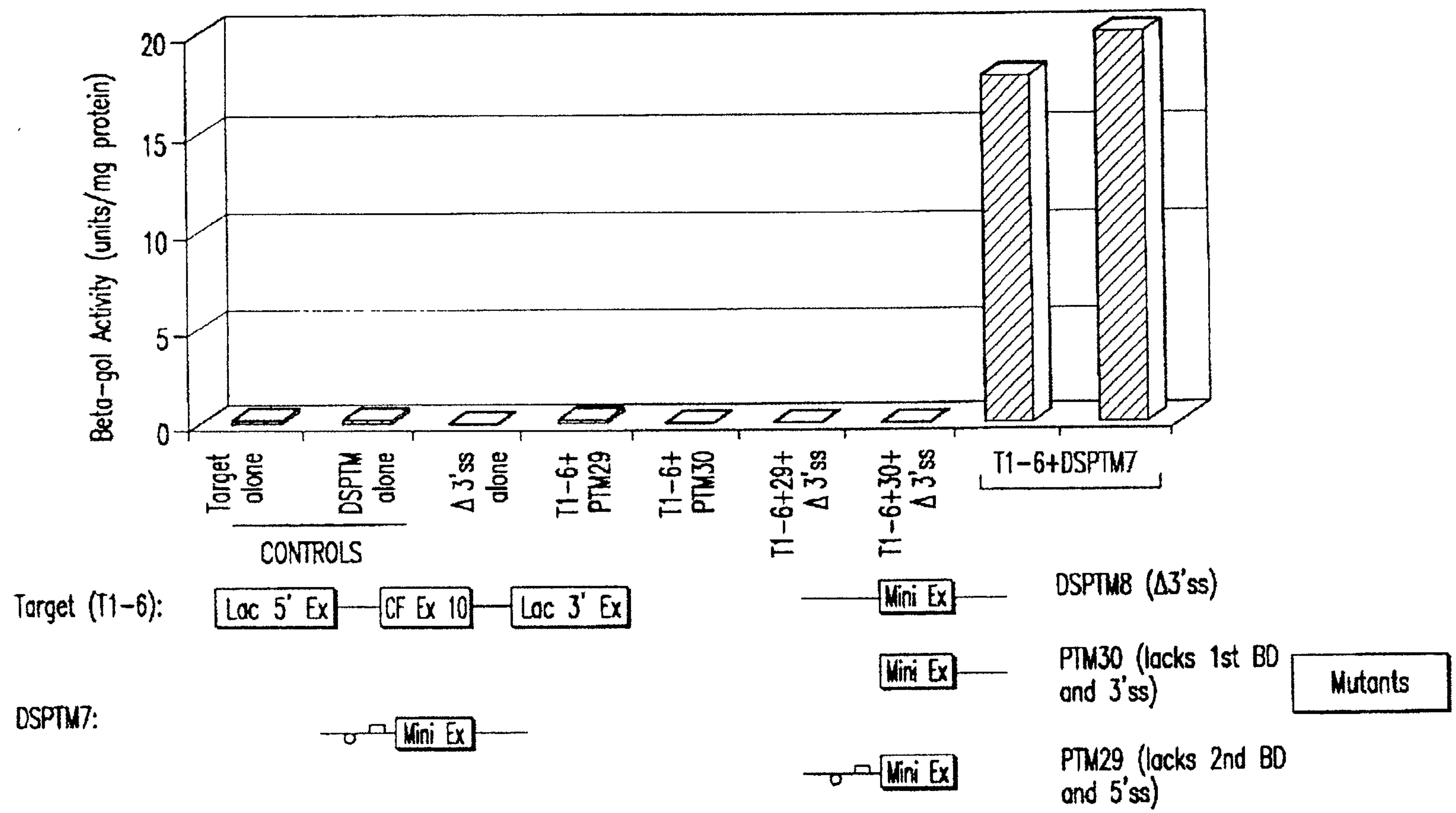
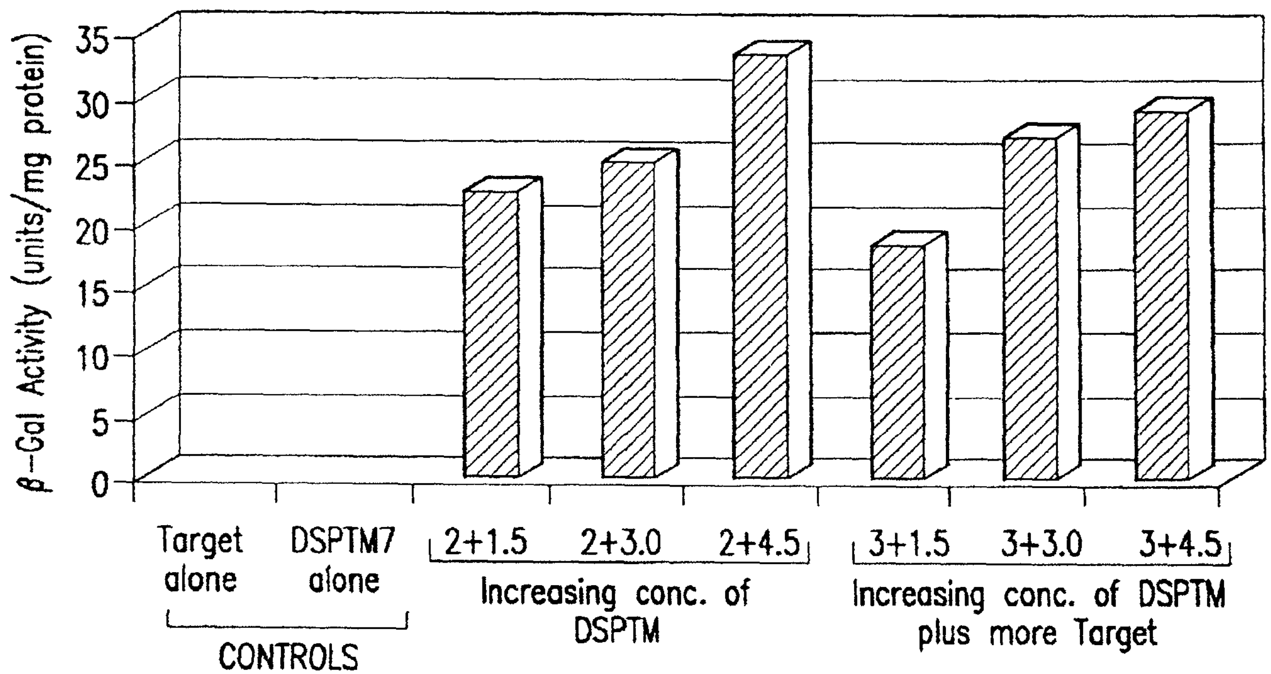


FIG.26

DOUBLE *TRANS*-SPLICING: TITRATION OF TARGET & PTM



The current level of beta-gal activity due to double *trans*-splicing is ~ 1-1.5% of the best single splice model (3' exon replacement)

FIG.27

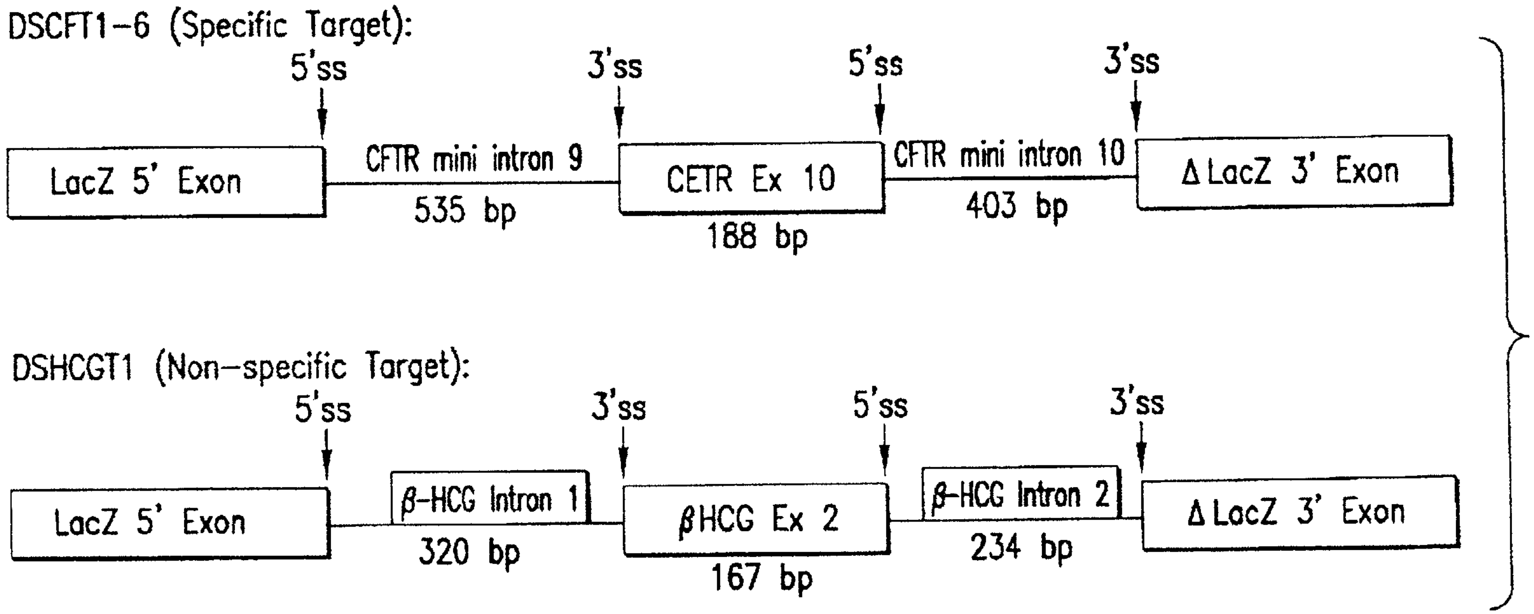

DSCFT1-6 (Specific Target):
5'ss
3'ss
5'ss
3'ss
LacZ 5' Exon
CFTR mini intron 9
535 bp
CETR Ex 10
188 bp
CFTR mini intron 10
403 bp
Δ LacZ 3' Exon
DSHCGT1 (Non-specific Target):
5'ss
3'ss
5'ss
3'ss
LacZ 5' Exon
β-HCG Intron 1
320 bp
βHCG Ex 2
167 bp
β-HCG Intron 2
234 bp
Δ LacZ 3' Exon

FIG.28

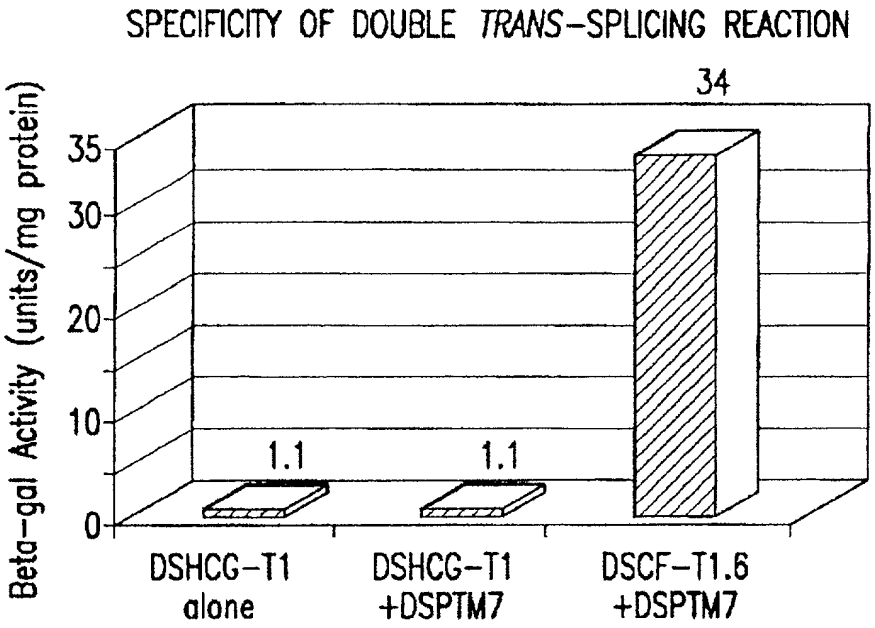

SPECIFICITY OF DOUBLE *TRANS*-SPLICING REACTION
Beta-gal Activity (units/mg protein)
35
30
20
10
0
34
1.1
1.1
DSHCG-T1 alone
DSHCG-T1 +DSPTM7
DSCF-T1.6 +DSPTM7

FIG.29

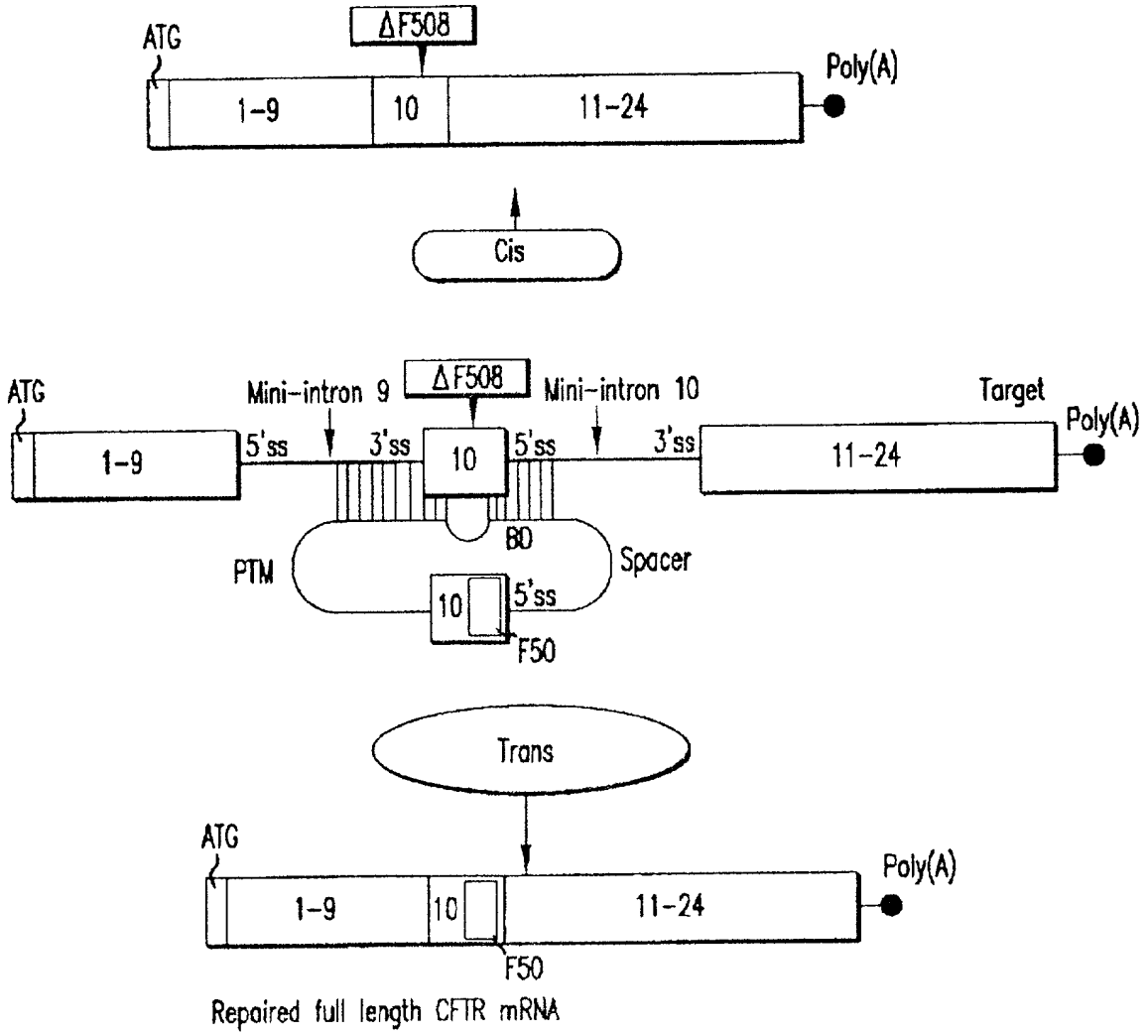
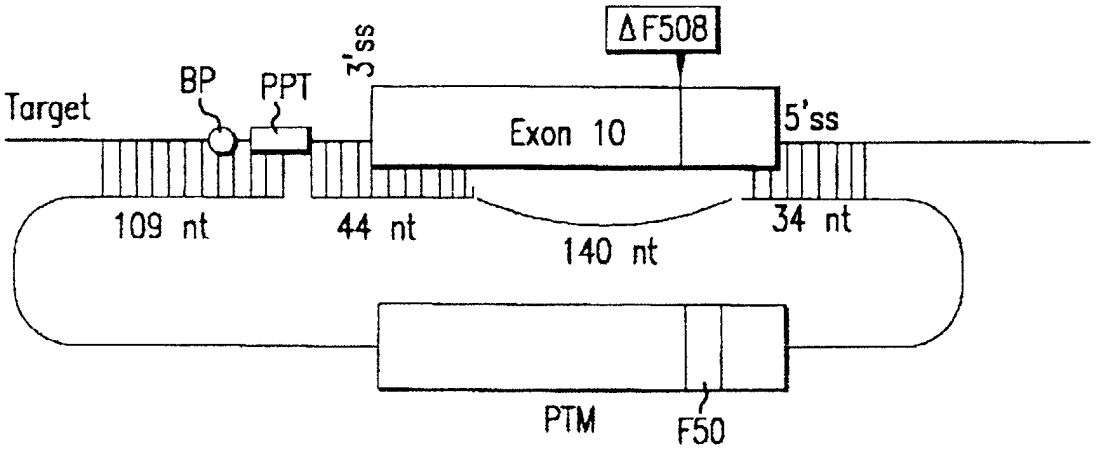

ATG
ΔF508
Poly(A)
1-9
10
11-24
Cis
ATG
Mini-intron 9
ΔF508
Mini-intron 10
Target
Poly(A)
1-9
5'ss
3'ss
10
5'ss
3'ss
11-24
B0
PTM
Spacer
10
5'ss
F50
Trans
ATG
Poly(A)
1-9
10
11-24
F50
Repaired full length CFTR mRNA

FIG.30

PTM with a long binding domain masking
two splice sites and part of exon 10
in a mini-gene target



ACGAGCTTGCTCATGATGATCATGGGCGAGTTAGAACCAAGTGAAGGCAAGATCAAACATTCCG
GCCGCATCAGCTTTTGCAGCCAATTCAGTTGGATCATGCCCGGTACCATCAAGGAGAACATAAT
CTTCGGCGTCAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAGGCCTGTCAGTTGGAGGAG

MCU in exon 10 of PTM
88 OF 192 (46%) bases in PTM exon 10 are not complementary to its binding domain (bold and underlined).

FIG.31

Sequence of a double
Trans–spliced product

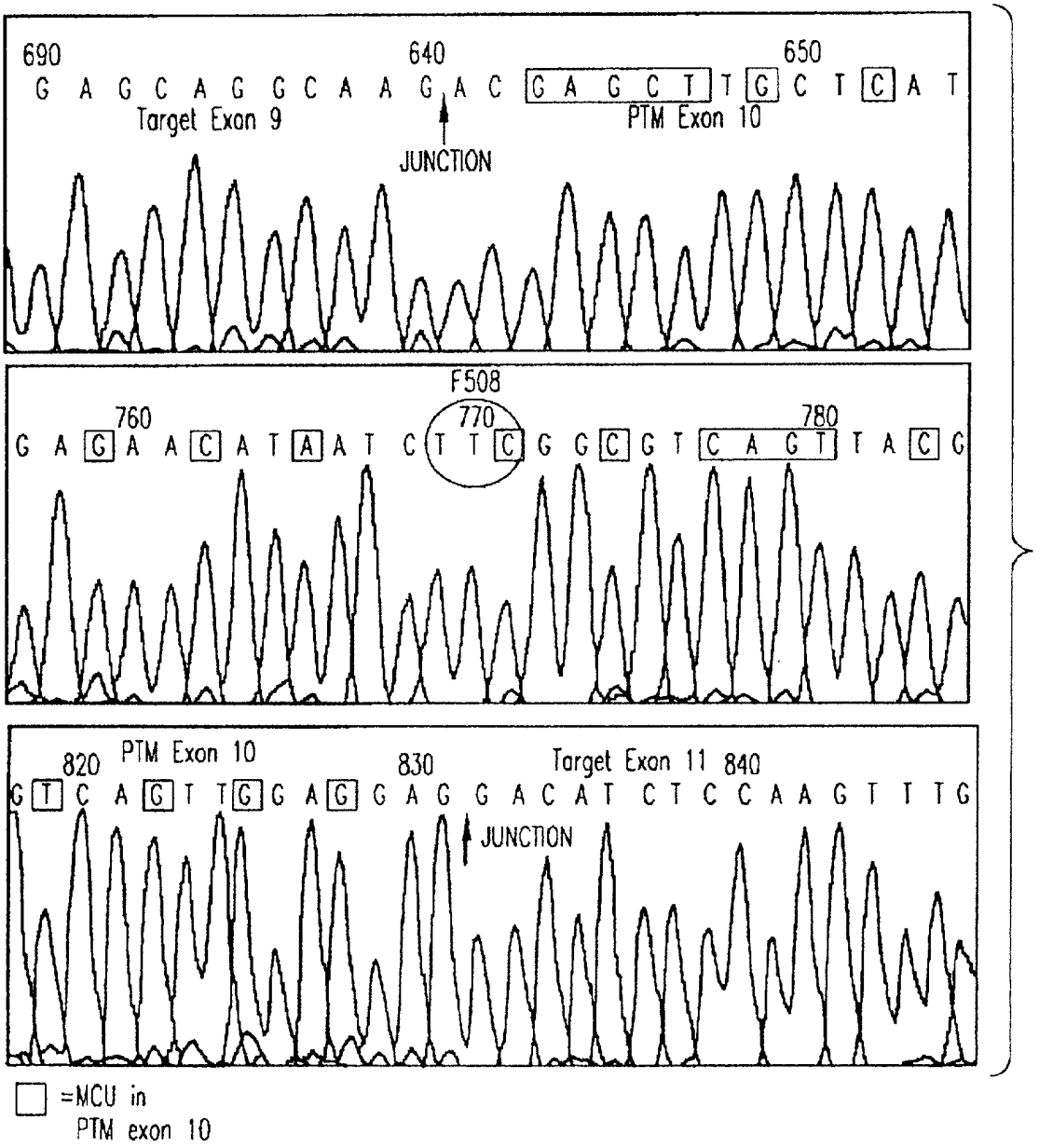


FIG.32

CF–TR Repair: 5' Exon–Replacement schematic diagram of a PTM binding to the splices site of intron 10 of a mini–gene target

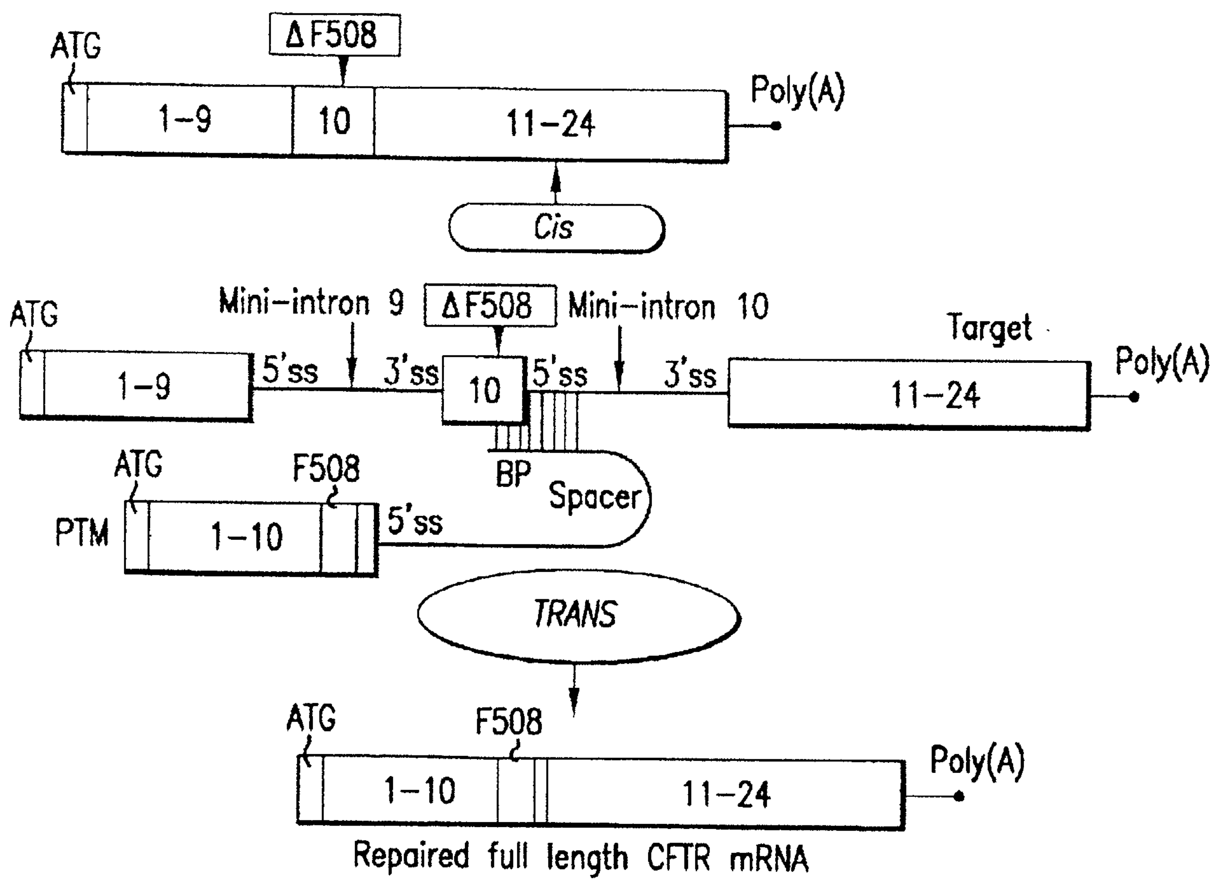


FIG.33

PTM with a short binding domain masking a single splice site in a mini-gene target.

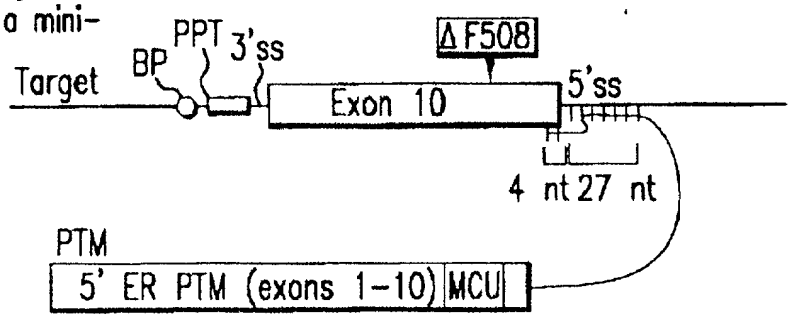


FIG.34A

PTM with a long binding domain masking two splice sites in a mini-gene target.

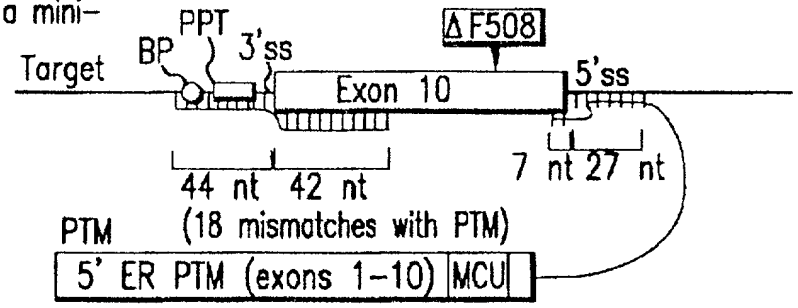


FIG.34B

PTM with a long binding domain masking two splice sites and the whole of exon 10 in a mini-gene target.

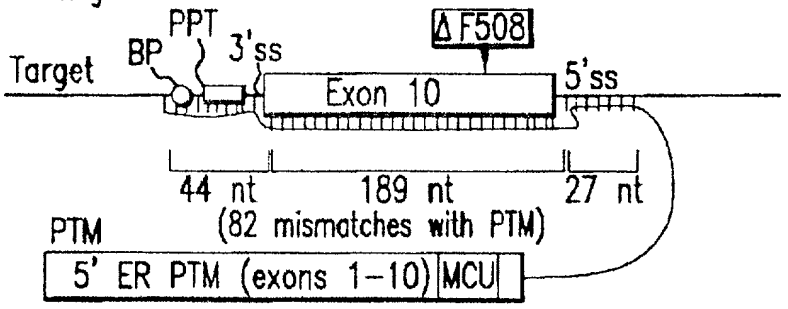
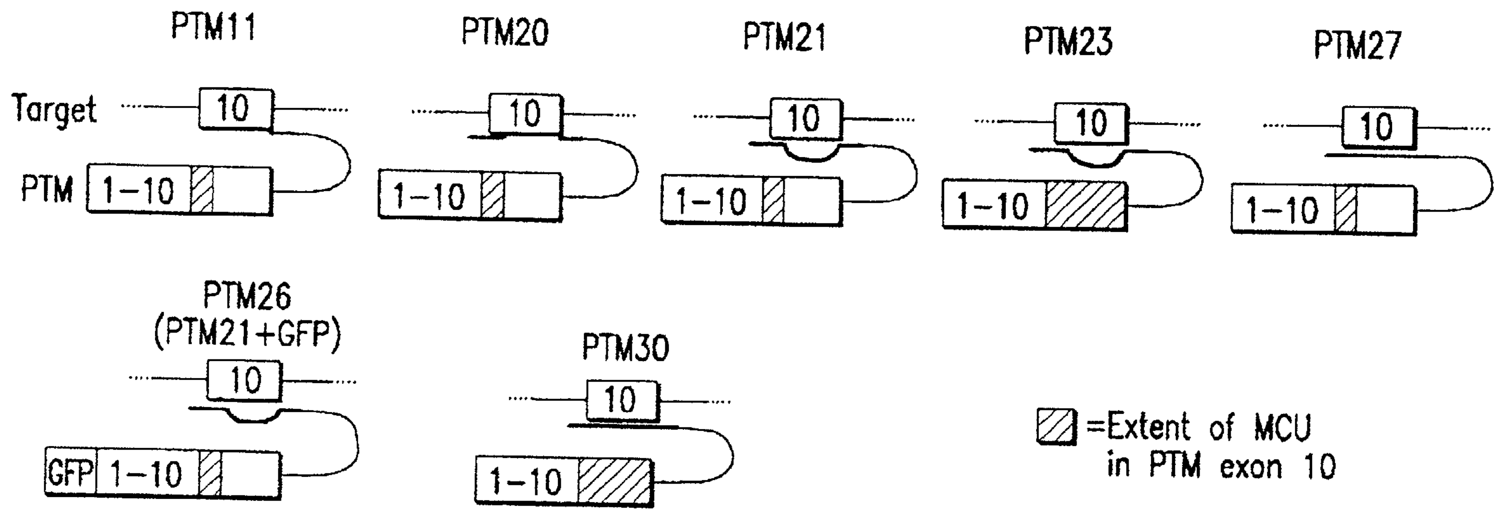


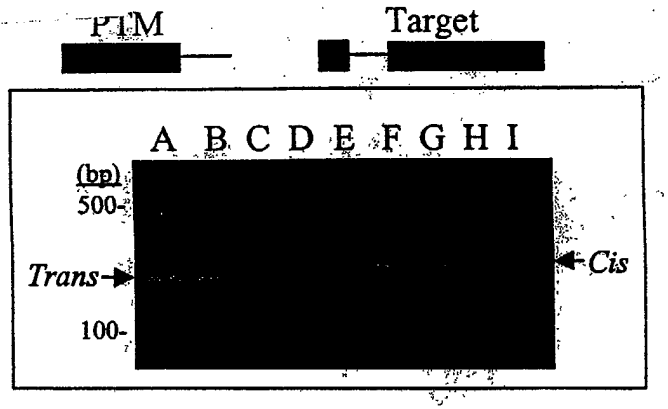
FIG.34C



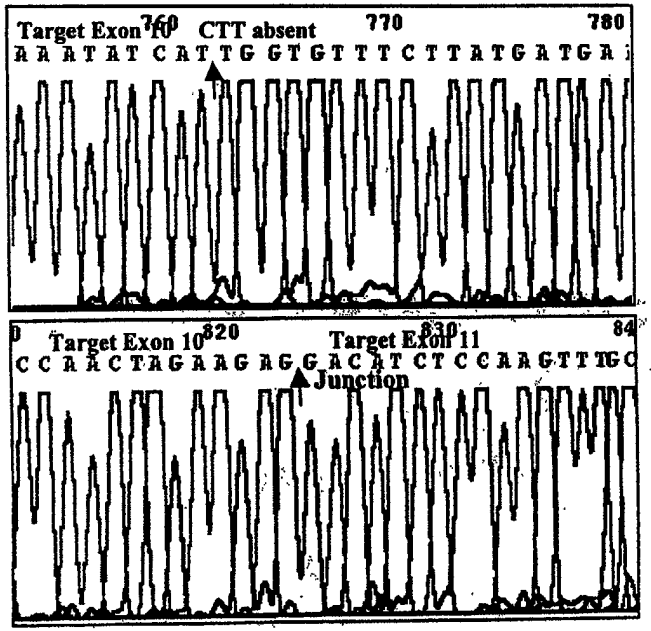
<u>MCU in exon 10 of PTM</u>
88 of 192 (46%) bases in PTM exon 10 are not complemetary to its binding domain.

ACGAGCTTGCTCATGATGATCATGGCCGAGTTAGAACCAAGTGAAGGCAAGATCAAACATTCCG
GCCGCATCAGCTTTTGCAGCCAATTCAGTTGGATCATGCCCGGTACCATCAAGGAGAACATAAT
CTTCGGCGTCAGTTACGACGAGTACCGCTATCGCTCGGTGATTAAGGCCTGTCAGTTGGAGGAG

FIG.35



A. *Cis*-spliced product
[Primers CF1 + CF111]



B. *Trans*-spliced product
[Primers CF93 + CF111]

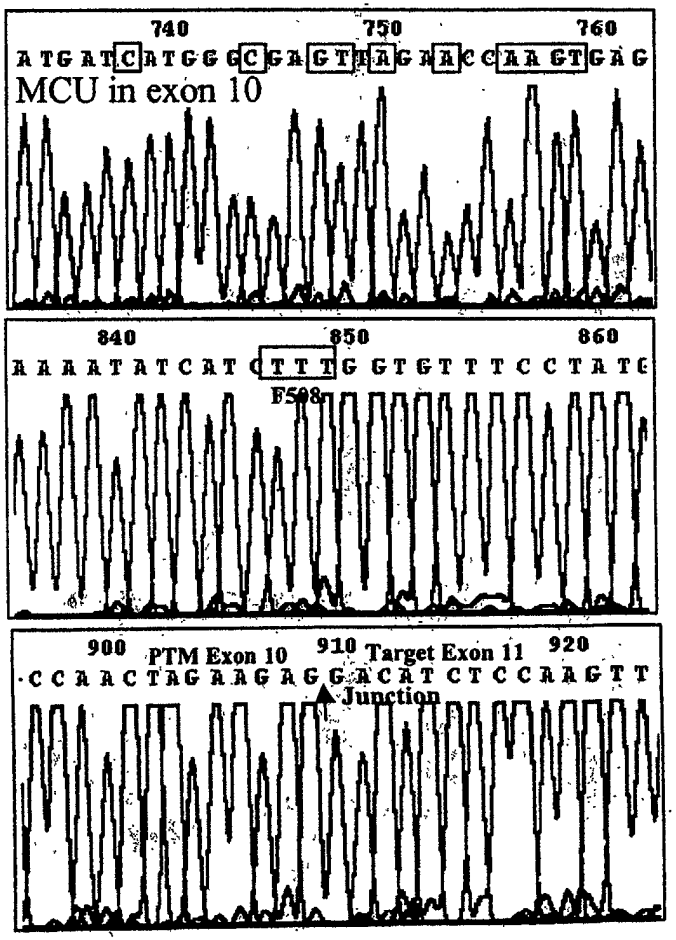


Figure 36

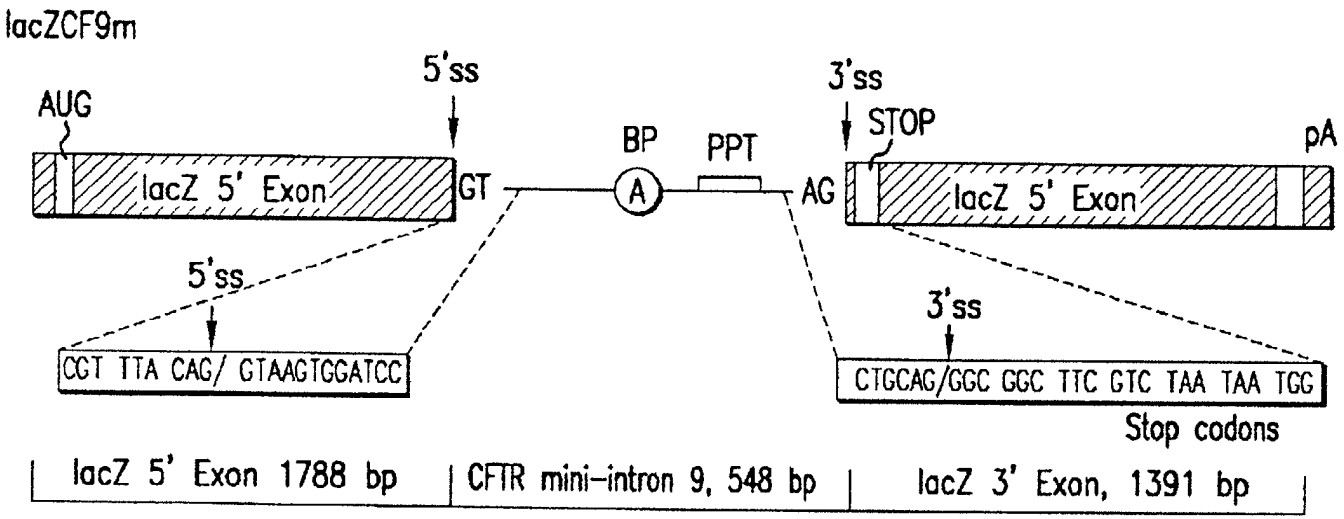

lacZCF9m
AUG
5'ss
lacZ 5' Exon
GT
BP
A
PPT
3'ss
STOP
AG
lacZ 5' Exon
pA
5'ss
CGT TTA CAG/ GTAAGTGGATCC
3'ss
CTGCAG/GGC GGC TTC GTC TAA TAA TGG
Stop codons
lacZ 5' Exon 1788 bp
CFTR mini-intron 9, 548 bp
lacZ 3' Exon, 1391 bp

FIG.37A

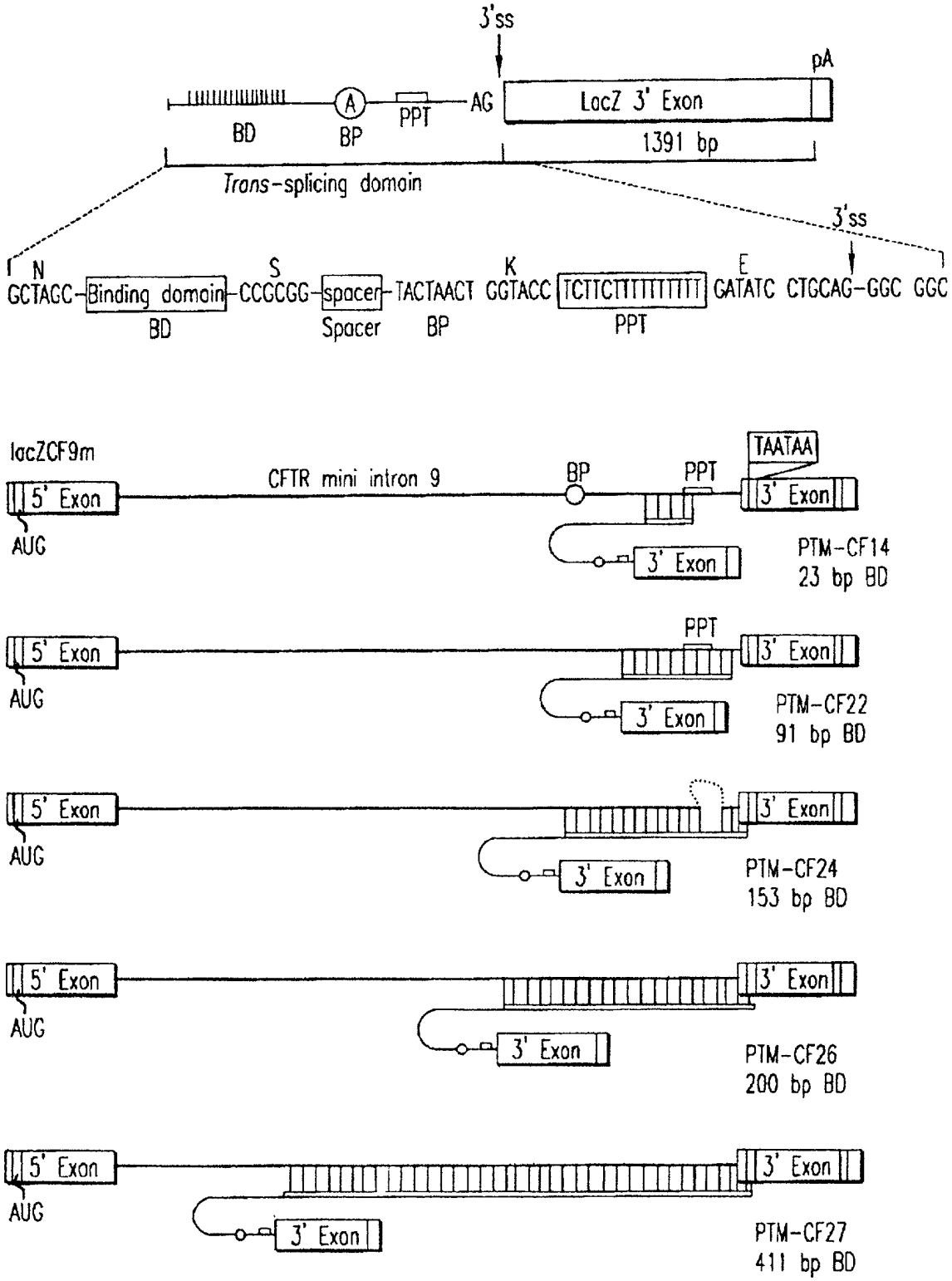

3'ss
pA
A
AG
LacZ 3' Exon
BD
BP
PPT
1391 bp
Trans-splicing domain
3'ss
N
S
K
E
GCTAGC-Binding domain-CCGCGG-spacer-TACTAACT GGTACC TCTTCTTTTTTTTT GATATC CTGCAG-GGC GGC
BD
Spacer
BP
PPT
lacZCF9m
5' Exon
CFTR mini intron 9
BP
PPT
TAATAA
3' Exon
AUG
PTM-CF14
23 bp BD
PTM-CF22
91 bp BD
PTM-CF24
153 bp BD
PTM-CF26
200 bp BD
PTM-CF27
411 bp BD

FIG.37B

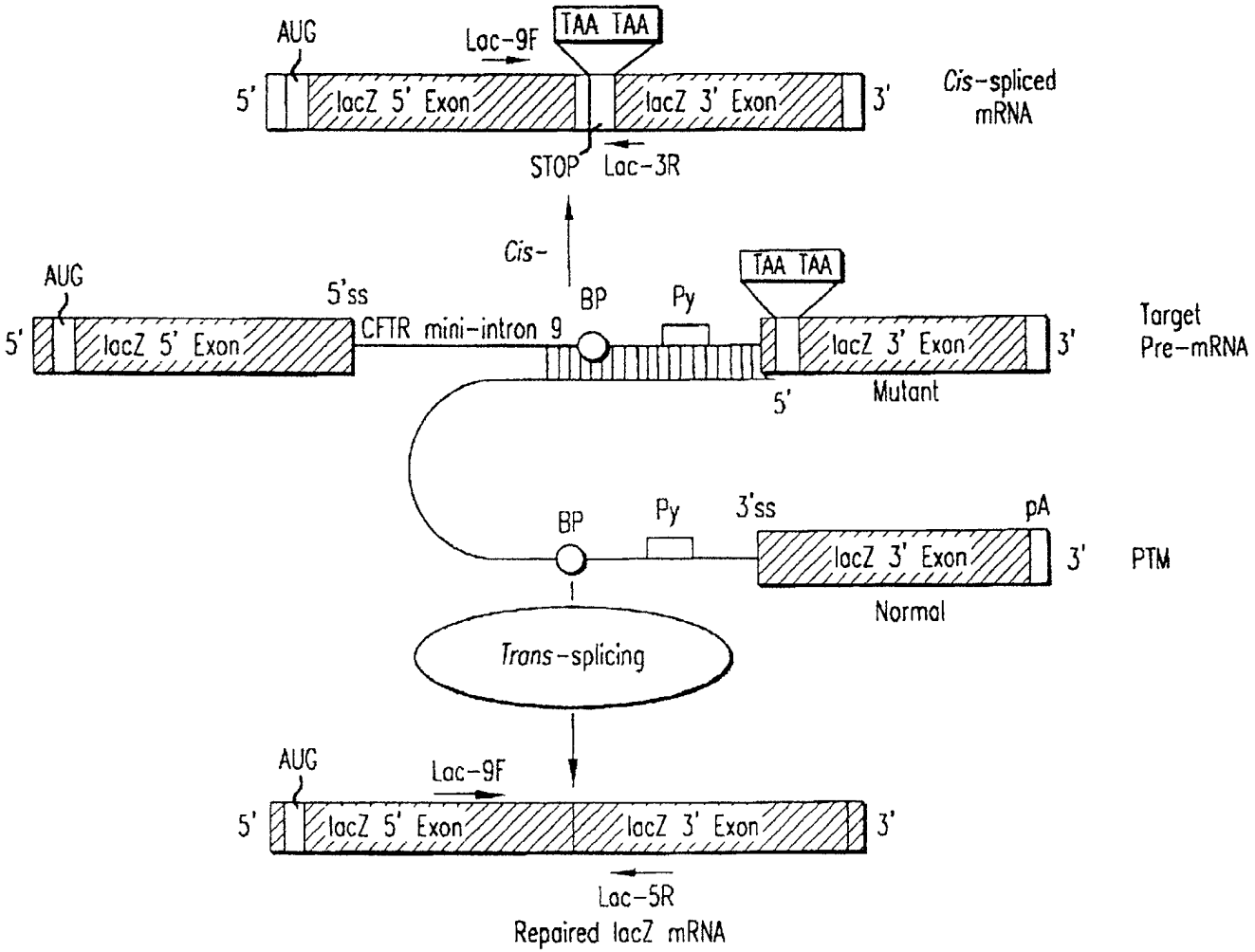
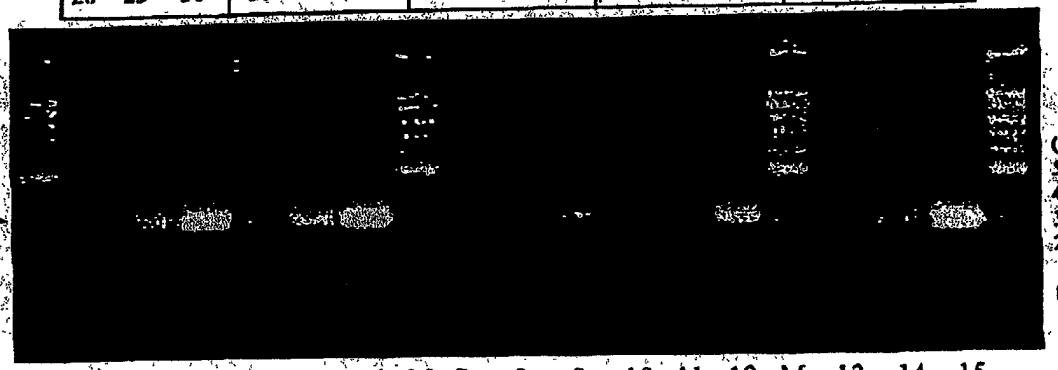


FIG.37C

A

Cis-splicing			*Trans*-splicing								
lacZCF9m + PTM-CF14									lacZCF9		
25 ng			50 ng			100 ng			200 ng			25 ng		
20	25	30	20	25	30	20	25	30	20	25	30	20	25	30

Total RNA
PCR cycles



Cis-spliced
(303 bp)

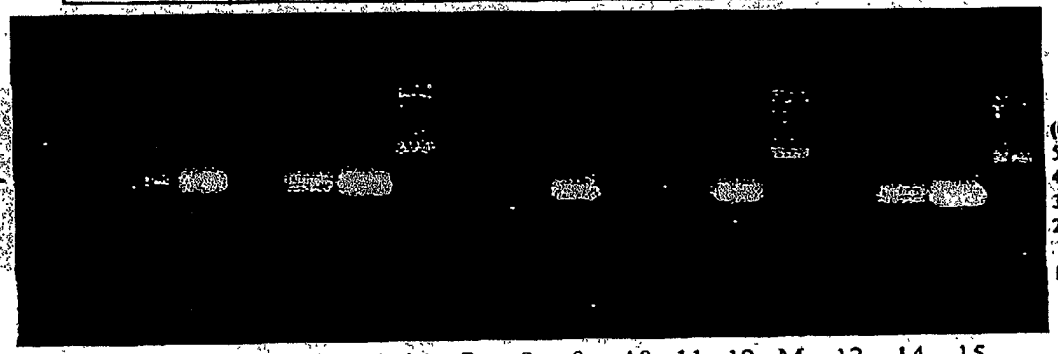
Trans-spliced
(299 bp)

1 2 3 4 5 6 M 7 8 9 10 11 12 M 13 14 15

Figure 38A

Cis-splicing			*Trans*-splicing								
lacZCF9m + PTM-CF24									lacZCF9		
25 ng			50 ng			50 ng			100 ng			50 ng		
20	25	30	20	25	30	20	25	30	20	25	30	20	25	30

Total RNA
PCR cycles



Cis-spliced
(303 bp)

Trans-spliced
(299 bp)

1 2 3 4 5 6 M 7 8 9 10 11 12 M 13 14 15

B

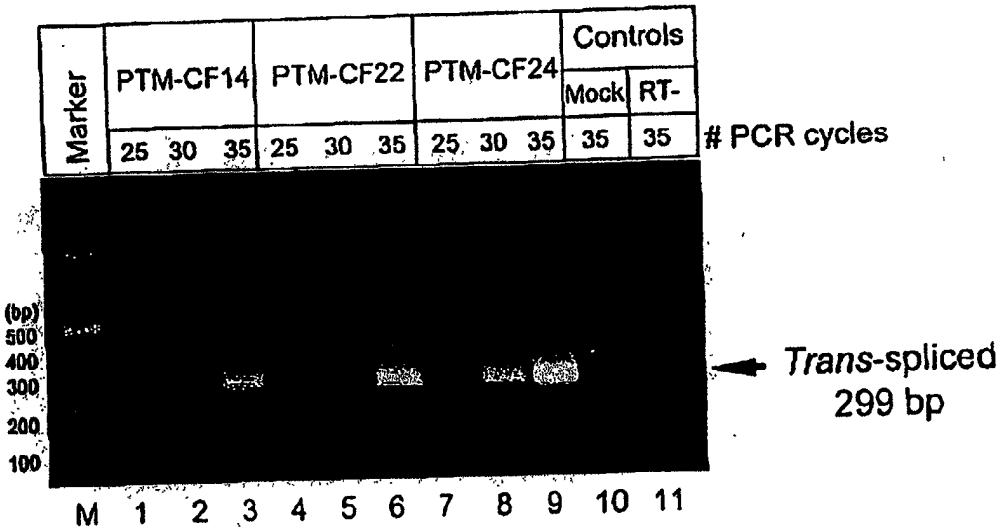

Marker
PTM-CF14
PTM-CF22
PTM-CF24
Controls
Mock
RT-
25 30 35 25 30 35 25 30 35 35 35
PCR cycles
(bp)
500
400
300
200
100
Trans-spliced
299 bp
M 1 2 3 4 5 6 7 8 9 10 11

Figure 38B

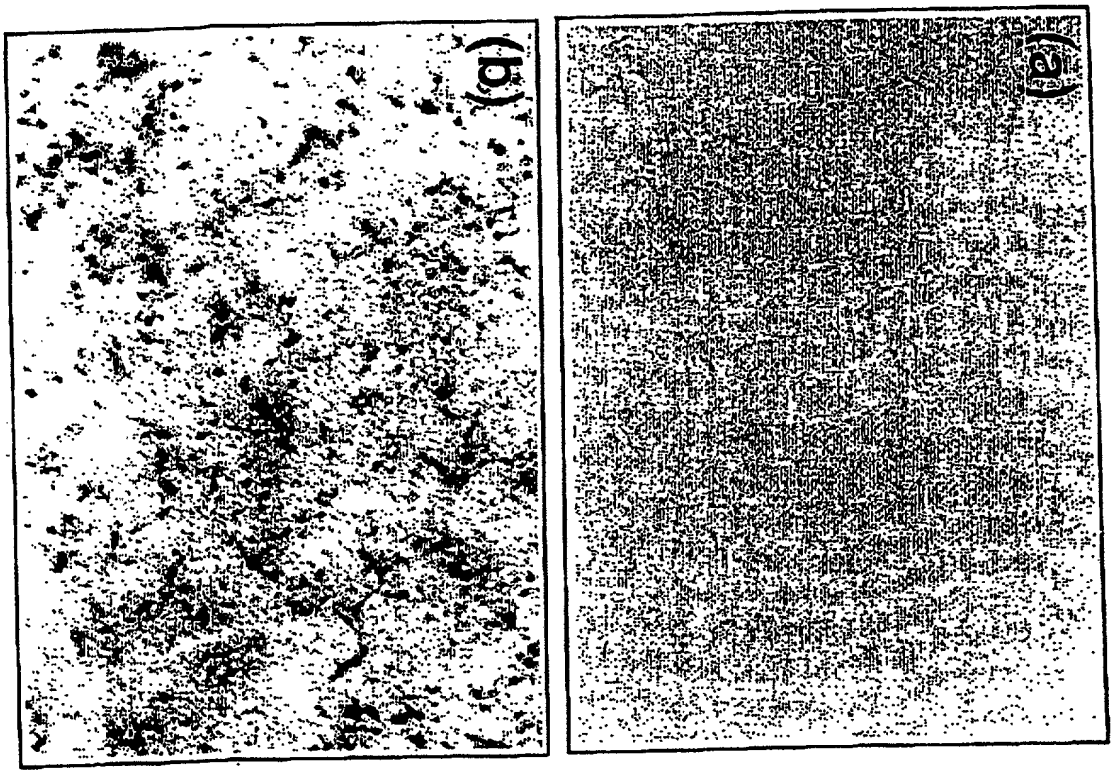


Figure 40A

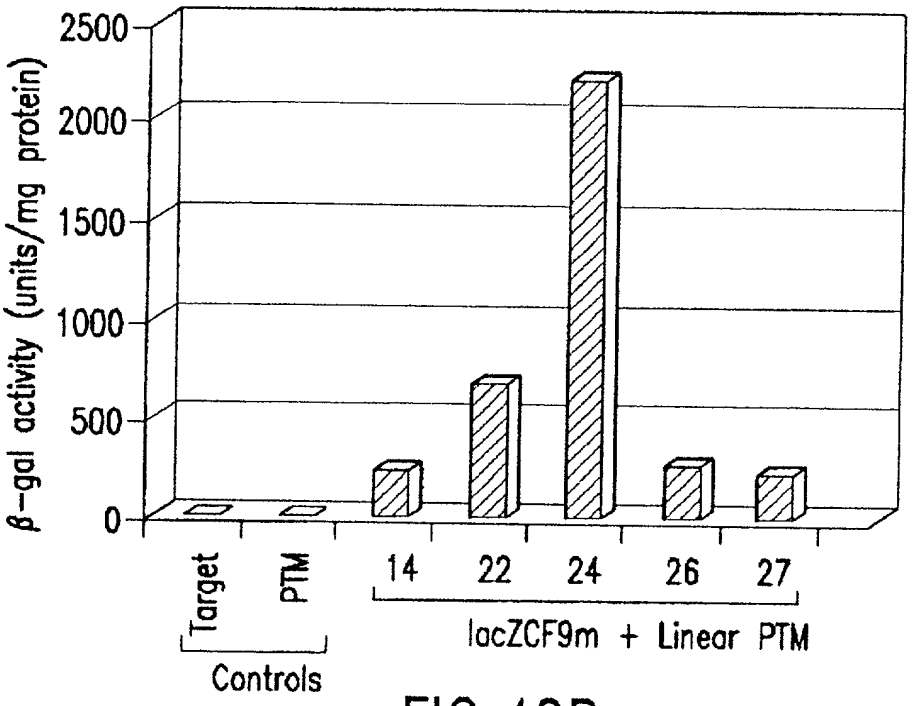


FIG.40B

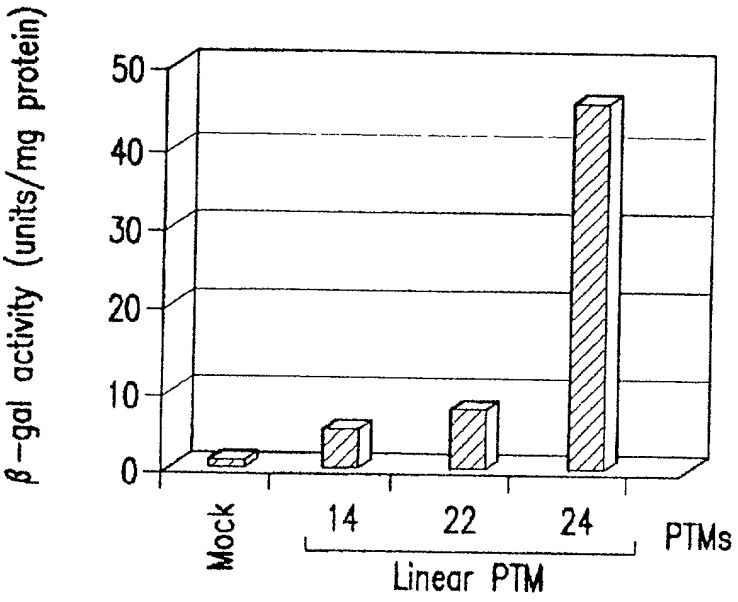
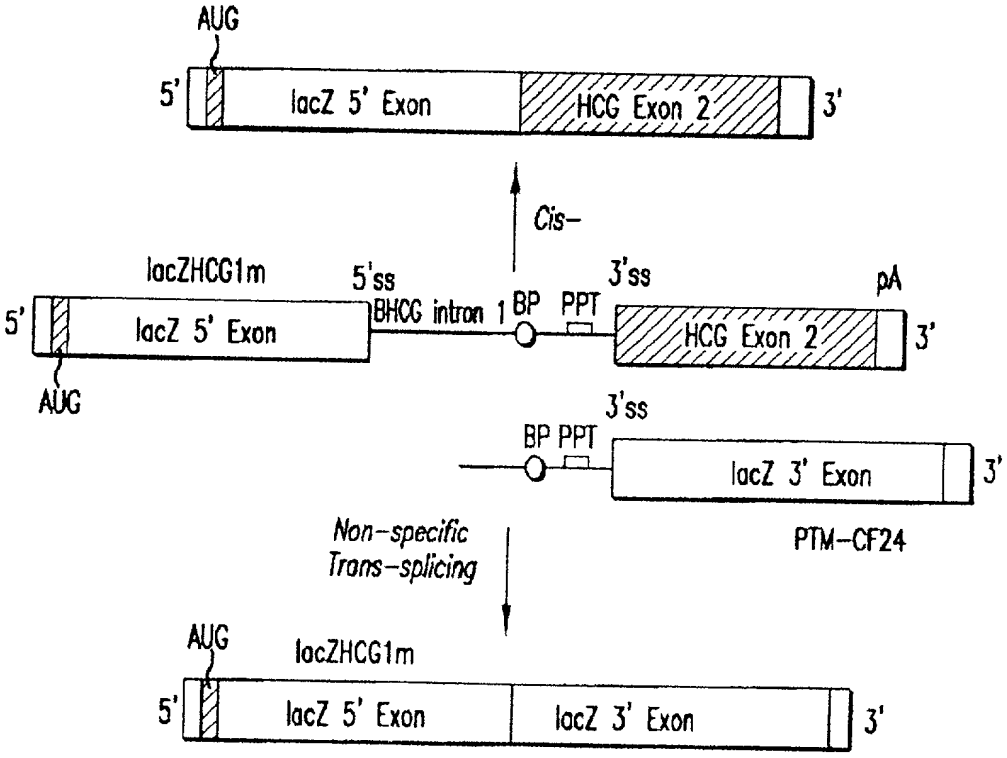


FIG.40C



Repaired lacZ mRNA

FIG.41A

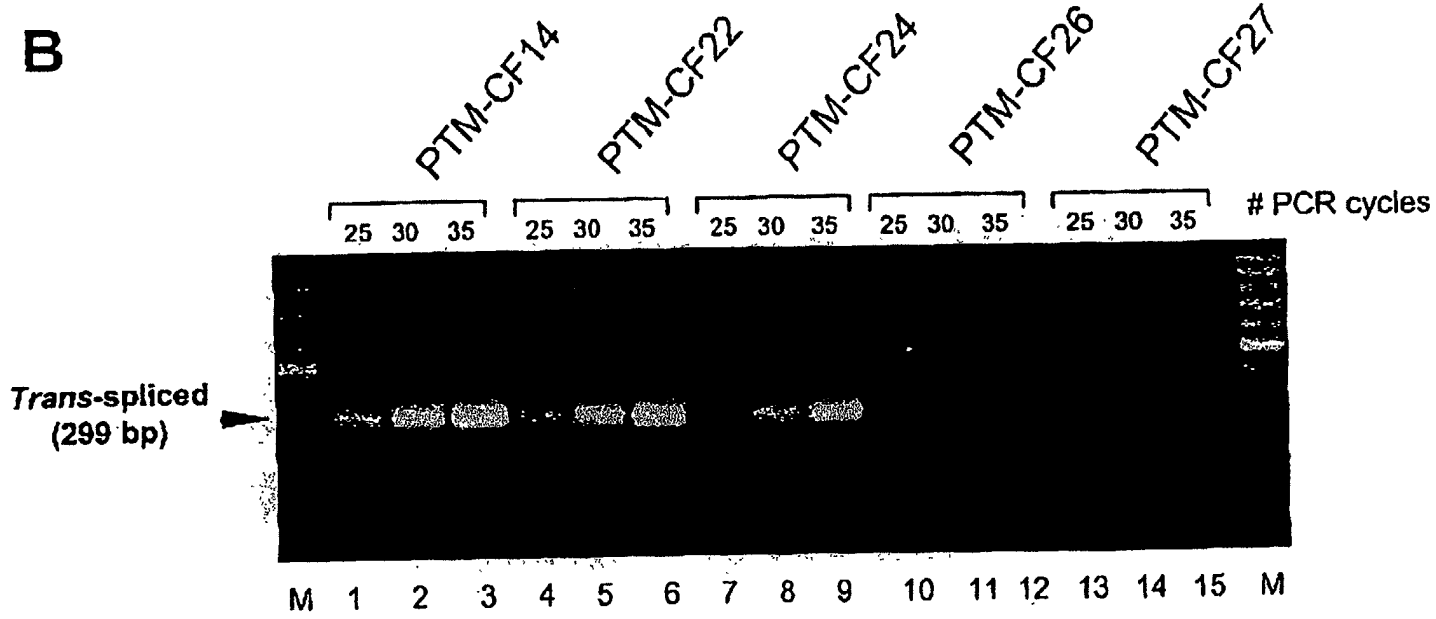

B
PTM-CF14
PTM-CF22
PTM-CF24
PTM-CF26
PTM-CF27
25 30 35
25 30 35
25 30 35
25 30 35
25 30 35
PCR cycles
Trans-spliced
(299 bp)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M

Figure 4B

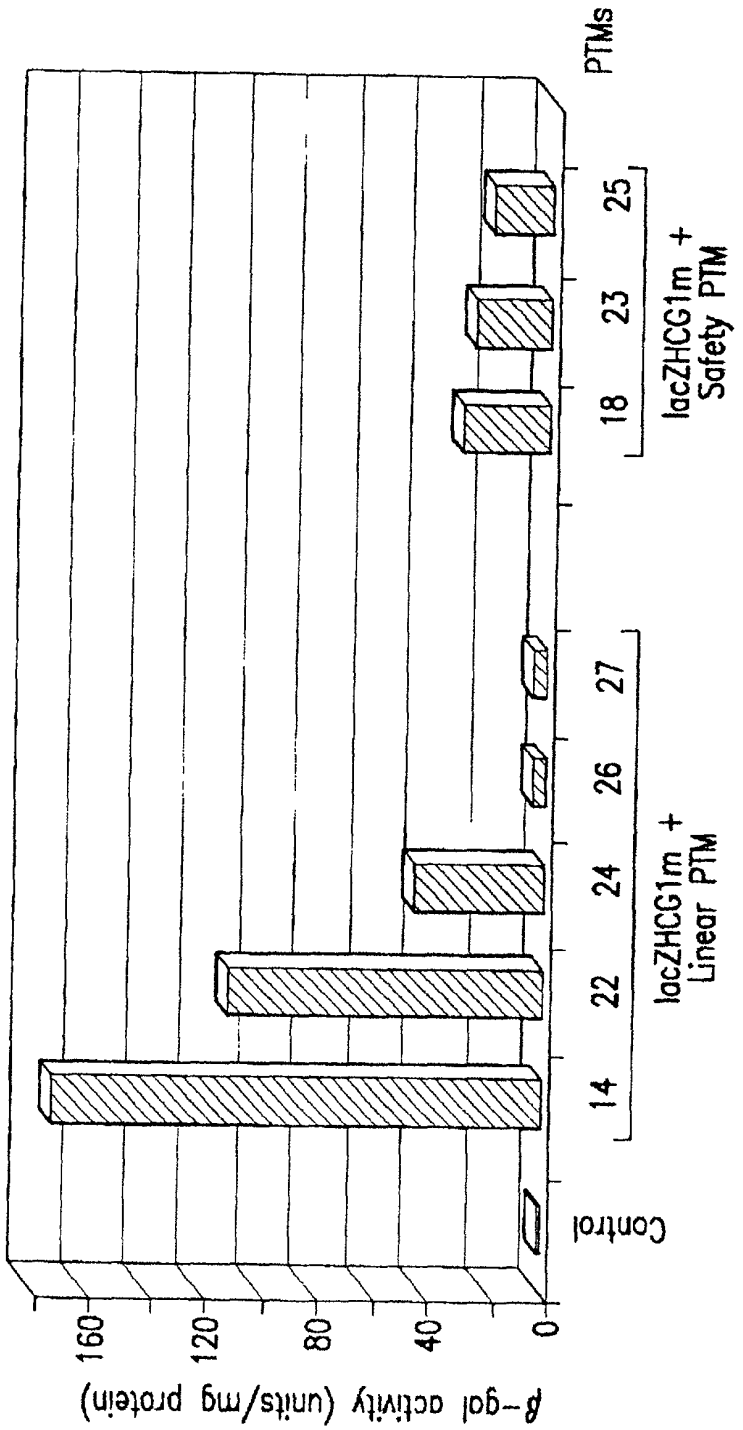

β-gal activity (units/mg protein)
0
40
80
120
160
Control
14
22
24
26
27
lacZHCG1m + Linear PTM
18
23
25
lacZHCG1m + Safety PTM
PTMs

FIG.41C

153 bp PTM24 Binding Domain:

Nhe I
GCTAGC-AATAATGACGAAGCCGCCCCTCACGCTCAGGATTCACTTGCCTCCAATTATCATCCTAAGCAGAAGTGTATA

153 bp BD underlined

TTCTTATTTGTAAAGATTCTATTAACTCATTTGATTCAAAATATTTAAAATACTTCCTGTTTCACCTACTCTGCTATGC

Sac II
AC-CCGCGG

FIG.43A

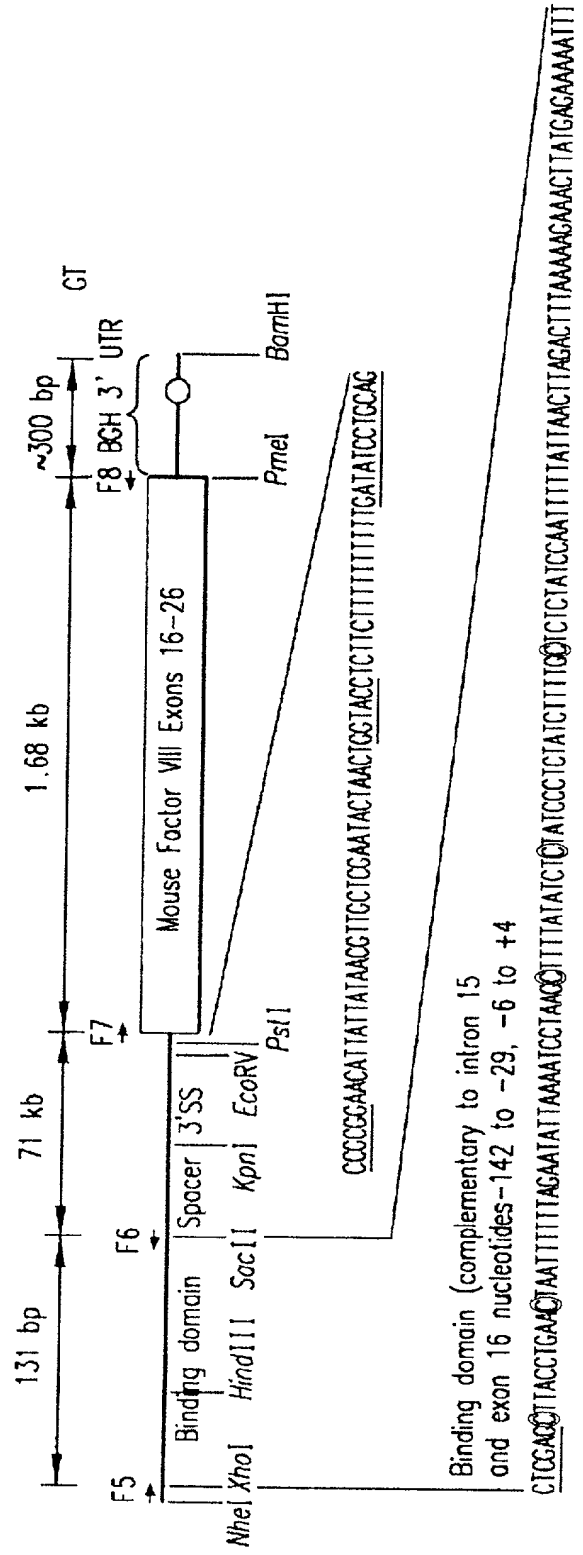

131 bp
71 kb
1.68 kb
~300 bp
F5
F6
F7
F8
Binding domain
Spacer
3'SS
Mouse Factor VIII Exons 16-26
BGH 3' UTR
GT
NheI XhoI HindIII SacII KpnI EcoRV PstI PmeI BamHI
CCCCCGAACATTATATAACCTTGCTCTCCAATACTAACTGGTACCTCTCTTTTTTTTTTTGATATCCTGCAG
Binding domain (complementary to intron 15
and exon 16 nucleotides -142 to -29, -6 to +4
CTCGAGGTTACCTGAAGTAATTTTTTAGAATATTAAAATCCTAAGTTTTATATCTGATCCCTCTATCTTTTGGTCTCTATCCAATTTTTATTAACTTAGACTTTAAAAAGAAACTTATGAGAAAAATTT

FIG.44A

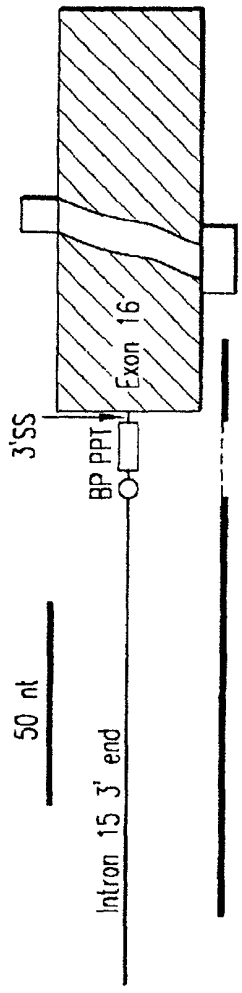


FIG.44B

NcoI
CCATGGTCGACGTTAGCCCCACGTTCTGCTTCACTCTCCCCATCTCCCCCCCCTCCCCACCCCCAATT

TTGTATTTATTTATTTTTTAATTATTTTGTGCAGCGATGGGGGCGGGGGGGGGGGGGGGCGCGCGCCAGGC

GGGGCGGGGCGGGGCCGAGGGGCGGGGCGGGGCGAGGCGGAGAGGTGCGGCGGCAGCCAATCAGAGCGGCGCG

CTCCGAAAGTTTCCTTTTATGGCGAGGCGGCGGCGGCGGCGGCCCTATAAAAAGCGAAGCGCGCGGCGGGCG

F13 XhoI
GGAGTCGCTGCGACGCTGCCTTCGCCCCGTGCCCAACCTCCGCCTCGAGCTTACCTGAACTAATTTTTTAGAA

HindII
TATTAAAATCCTAAGCTTTTATATCTCTATCCCTCTATCTTTTGCTCTCTATCCAATTTTTATTAACTTAGA

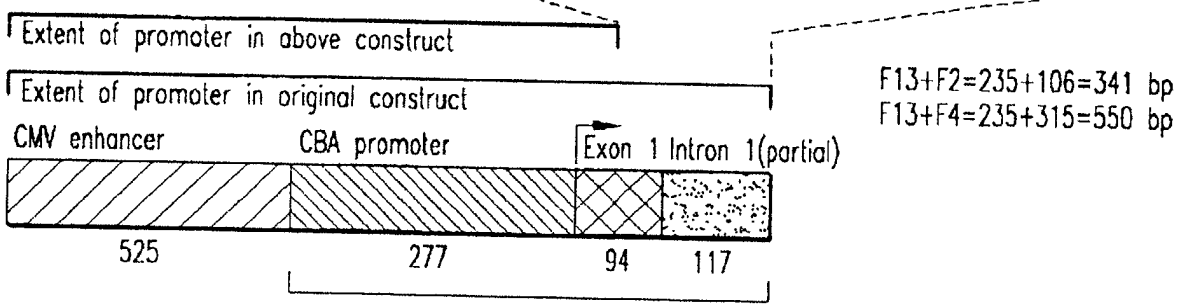
SacII
CTTTAAAAAGAAACTTATGAGAAAAATTTCCGCGGAACATTATTATAACGTTGCTCGAATACTAACTGGTAC

EcoRV PstI
CTCTTCTTTTTTTTTTGATATCCTGCAG

Chicken β-actin
Promoter

Nucleotide changes are shown in blue
Boxed=Cat box, TATA box
Boxed+Arrow=Transcription Start
Oval=Downstream elements
Bold=Binding domain
Italicized=Spacer+PPT+BP+AG dinucleotide

Sequence not included in construct
CGCCGCCTCGCCCCGCCCGCCCCGGCTCTGACTGACCGCGTTACTCCCACAGGTGAG
CGGGCGGGACGGCCCTTCTCCTCCGGGCTGTAATTAGCGCTTGGTTTAATCACGGCT
TGTTTCTTTTCTGTGGCTGCGTGAAAGCCTTGAGGGGCTCCGGGAGGAATTCGTA



F13+F2=235+106=341 bp
F13+F4=235+315=550 bp

FIG.44C

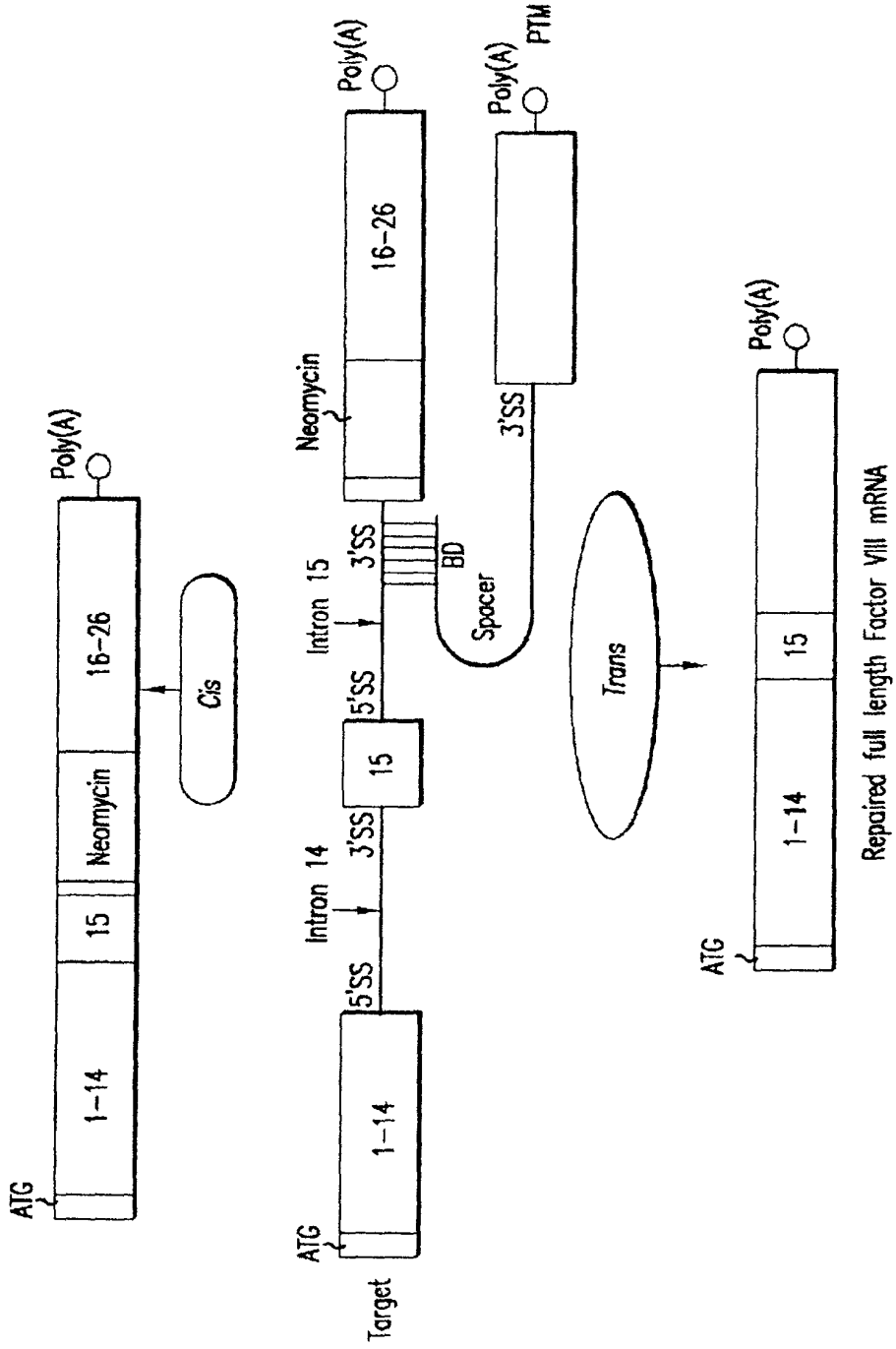
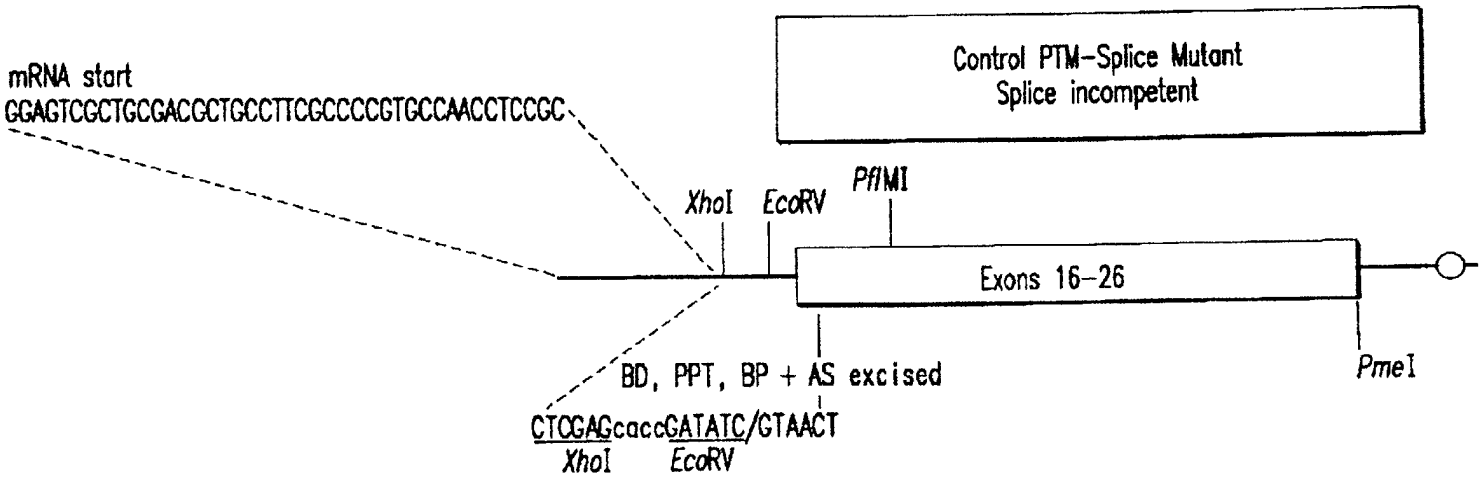

ATG
1-14
15
Neomycin
16-26
Poly(A)
Cis
Target
ATG
1-14
5'SS
Intron 14
3'SS
15
5'SS
Intron 15
3'SS
Neomycin
16-26
Poly(A)
BD
Spacer
3'SS
Poly(A)
PTM
Trans
ATG
1-14
15
Poly(A)
Repaired full length Factor VIII mRNA

FIG.44D



Method:
Excise TSD and part of exon 16 with
XhoI and PflMI and ligate in a PCR product that:
1) eliminates the TSD and splice acceptor site
2) inserts EcoRV adjacent to exon 16
3) restores the coding for exon 16

FIG.45

Repair of Factor VIII
Preliminary results from one experiment

FVIII activity in Exon 16 FVII-KO mice
after IV PTM-FVII intraportal infusion
(100μgDNA)(n=3)

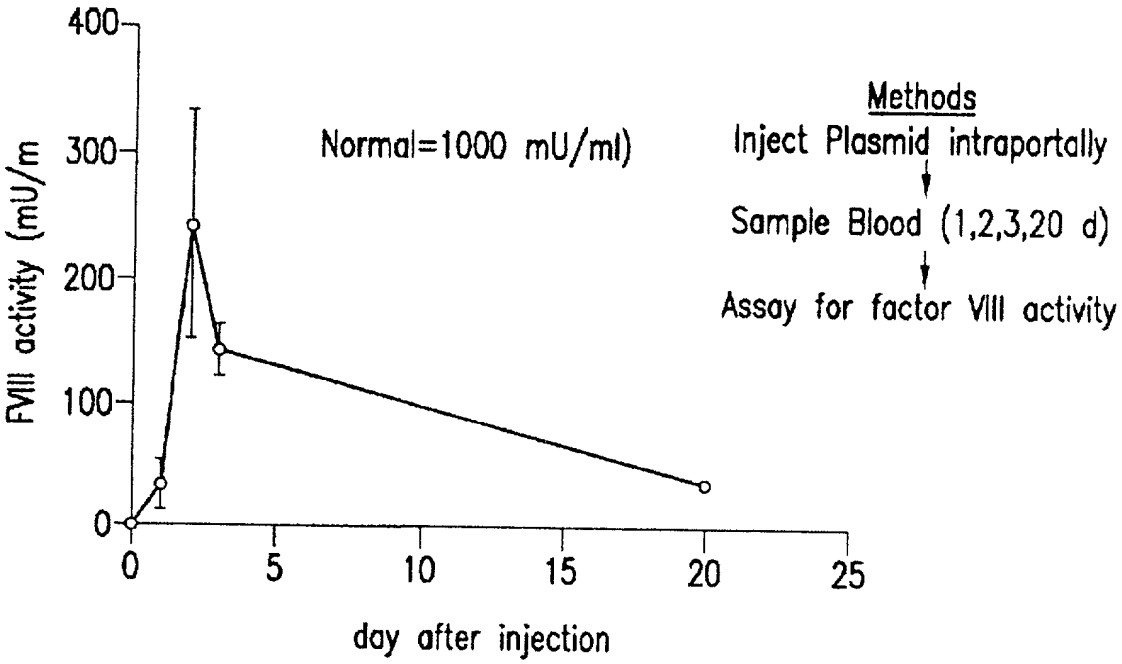


FIG.46

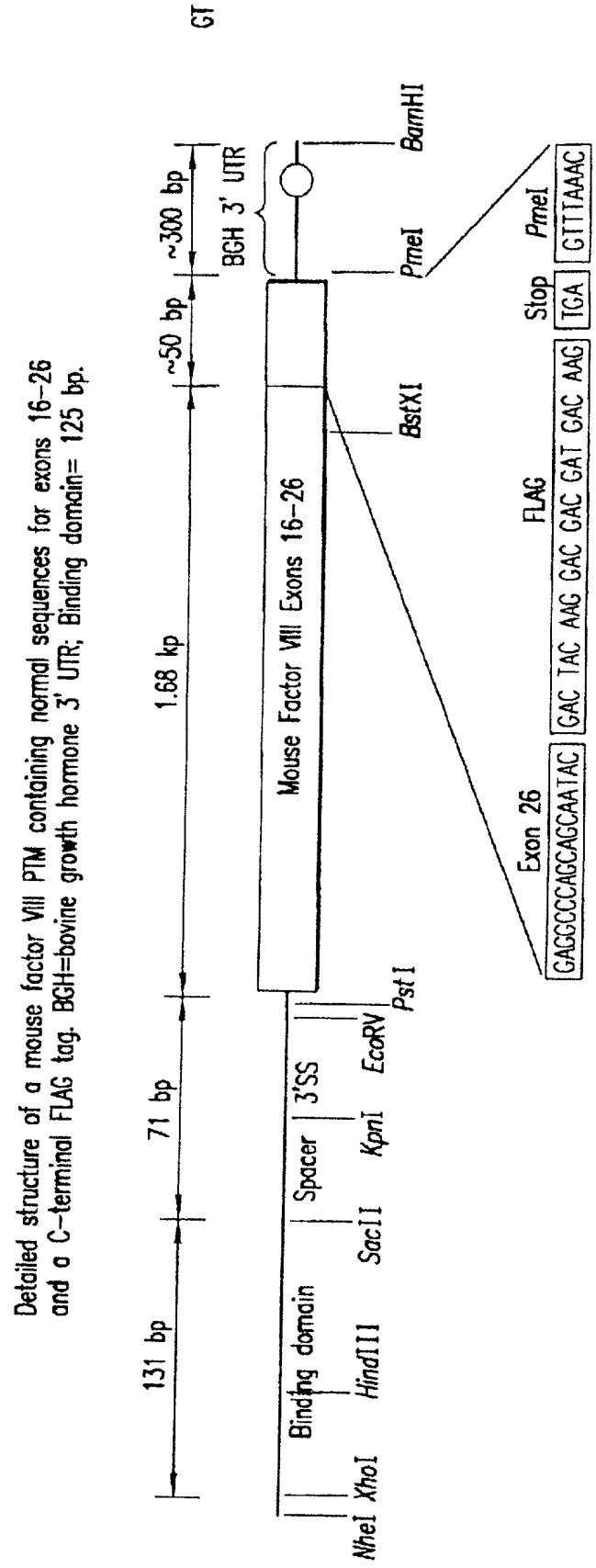
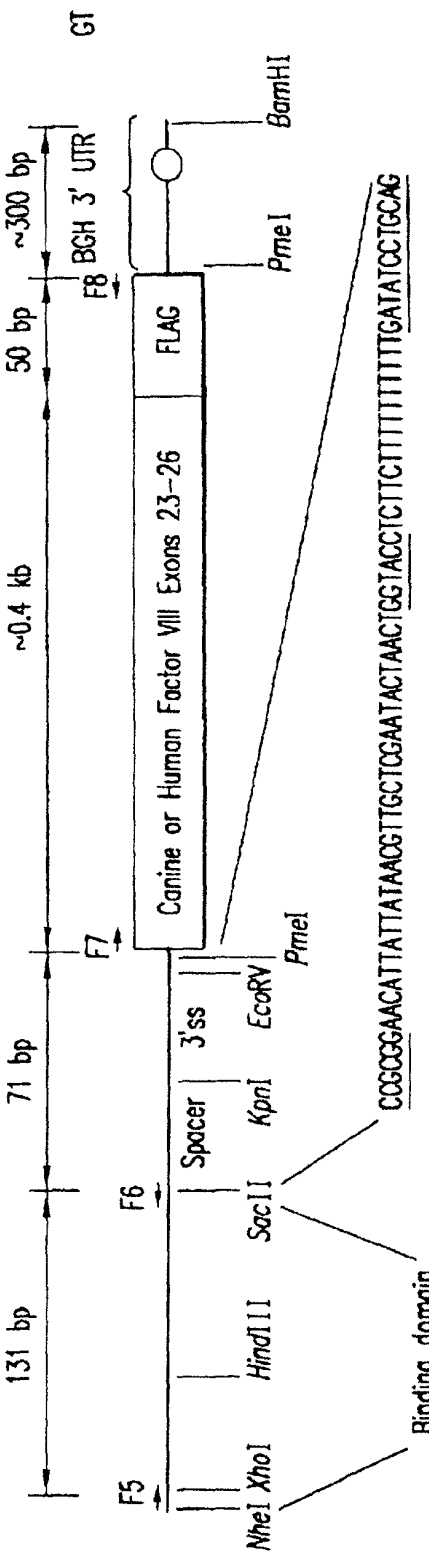

Detailed structure of a mouse factor VIII PTM containing normal sequences for exons 16-26
and a C-terminal FLAG tag. BGH=bovine growth hormone 3' UTR; Binding domain= 125 bp.
131 bp
71 bp
1.68 kp
~50 bp
~300 bp
GT
BGH 3' UTR
Binding domain
Spacer
3'SS
Mouse Factor VIII Exons 16-26
NheI
XhoI
HindIII
SacII
KpnI
EcoRV
PstI
BstXI
PmeI
BamHI
Exon 26
FLAG
Stop
PmeI
GAGGCCCAGGCAGCAATAC
GAC TAC AAG GAC GAC GAT GAC AAG
TGA
GTTTAAAC

FIG.47A



FLAG=C-terminal tag to be used to detect repaired factor VIII protein.

FIG.47B